SOME METHODOLOGICAL DEVELOPMENTS IN PHOSPHOLIPID CHEMISTRY & PHYSICO-CHEMICAL STUDIES OF Ca^{2+} INDUCED CHANGES IN CARDIOLIPIN VESICLES

By

RAKESH MOHAN HALLEN

DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY KANPUR

AUGUST, 1981

SOME METHODOLOGICAL DEVELOPMENTS IN PHOSPHOLIPID CHEMISTRY & PHYSICO-CHEMICAL STUDIES OF Ca^{2+} INDUCED CHANGES IN CARDIOLIPIN VESICLES

A Thesis Submitted
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

By

RAKESH MOHAN HALLEN

DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY KANPUR

AUGUST, 1981

CERTIFICATE I

Certified that the work presented in this thesis entitled, 'Some Methodological Developments in Phospholipid Chemistry and Physico Chemical Studies of Ca^{2+} Induced Changes in Cardiolipin Vesicles' by Mr. Rakesh Mohan Hallen, has been carried out under my supervision and not submitted elsewhere for a degree.

Pinaki Gupta-Bhaya

Professor P. Gupta-Bhaya
Department of Chemistry
I.I.T. Kanpur-16.

July, 1981

8-4-1982

DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY KANPUR

CERTIFICATE II

This is to certify that Mr. Rakesh Mohan Hallen has satisfactorily completed the following courses in partial fulfilment for Ph.D. programme.

Chm	500	Mathematics for Chemists
Chm	501	Advanced Organic Chem. I
Chm	511	Physical Organic Chem.
Chm	521	Chemical Binding
Chm	523	Chemical Thermodynamics
Chm	524	Modern Physical Methods
Chm	541	Advanced Inorganic Chem. I
Chm	581	Basic Biological Chem.
CS	609	Introductory Programming
Phy	432	Quantum Mechanics I
Phy	452	Electricity and Magnetism
Math	530	Topology
Math	601	Mathematical Methods

Mr. Rakesh Mohan Hallen was admitted to the candidacy for Ph.D. degree in May, 1976 after he successfully completed the written and oral comprehensive examinations.

U.C. Aggarwala

(U.C. Aggarwala)
Professor and Head
Department of Chemistry
I.I.T. Kanpur-208016.

P. C. Nigam

(P.C. NIGAM)
Convener, DPGC
Department of Chemistry
I.I.T. Kanpur-208016.

STATEMENT

I hereby declare that the work submitted in this thesis entitled 'Some Methodological Development in Phospholipid Chemistry and Physico-Chemical Studies of Ca^{2+} Induced Changes in Cardiolipin Vesicles' has been carried out by me under the supervision of Professor P. Gupta-Bhaya.

In keeping with scientific tradition, wherever work done by others has been utilized, due acknowledgement has been made.

Rakesh Mohan Hallen

ACKNOWLEDGEMENTS

For me it is a rare opportunity to put my gratitudes on record, since it is at my expense only I wish to exploit it fully.

I am grateful to the millions of my toiling fellow countrymen who contribute towards the maintenance of this grand Institute, so comfortable and calm. I am also grateful to those Americans who were instrumental in introducing certain values in this Institute which made possible my casual and bohemian existence.

It is perhaps customary to begin this page with a verbose acknowledgement to one's thesis supervisor, but I can only say that my gratitude towards Dr. Pinaki Gupta Bhaya is beyond words. But for his perseverance and understanding attitude for all these years this thesis would never have taken its present shape. We have had rather strong differences in opinion but it is perhaps mainly due to his tolerance that we have got along so well. Above all he granted me the freedom which I so desperately needed.

Besides I am grateful to -

Pa and Ma for their indulgent patience all these years.

Dinesh Kumar and Pratibha Chopra my lab. mates for sharing some extreme moments with me.

Comrades Ravi, Kumar, Shankar, Tank, Naik, Ramaswamy, Raghu, Keshavmurthy, Subbu, Swami, Vish, Ballal, Somnath, Sanjeeve, Muddhu, CPK, VVK and many others for the pleasent comradeship.

Mr. Jha of Chemistry workshop, Mr. Das of electron microscopy lab., Mr. Sharma and Mr. Subramanian of glass blowing shop, Mr. Vishal Saxena of Central Instrumentation Services, and Mr. Bakshi Singh of Refrigeration workshop for the timely help in many a moments of crisis amidst the widespread lethargy.

To C.S.I.R., New Delhi and D.A.E. Bombay for the research grants to Dr. Gupta Bhaya, which are extremely helpful amidst the rampant corruption.

Mr. Abraham, Mr. Dilip and Mr. Bishabhar Nath for the help in putting the thesis in print.

And lastly many individuals around, most of whom I do not know the name even, but who have inspired, consoled and encouraged me at times in ways beyond understanding.

TABLE OF CONTENTS

SUMMARY

This thesis embodies the results of an attempt to further study the Ca^{2+} ion induced transformations in cardiolipin vesicle solutions within our institutional and instrumental facilities limitations. Calcium ion induced phase changes in cardiolipin and phosphatidylserine vesicles solutions have been studied earlier using X-ray diffraction, freeze fracture electron microscopy, differential scanning microcalorimetry nuclear magnetic resonance and autocorrelation laser light scattering techniques. We have however attempted a study using mainly a UV-VIS spectrophotometer.

The thesis begins with a brief review of the physical chemistry of phospholipids and phospholipid aqueous dispersions (Chapter I). The next three chapters describe the three methodologies developed in order to be able to carry out the proposed studies.

Chapter II describes a colorimetric method developed to determine the phospholipid concentrations in aqueous dispersions directly. This method is based on the formation of a phospholipid molybdenum blue complex in the aqueous phase which is extractable with some organic solvents and the lipid concentration can be determined spectrophotometrically. The optimal conditions for the preparation of the reagent and estimation are reported together with the specificity of the

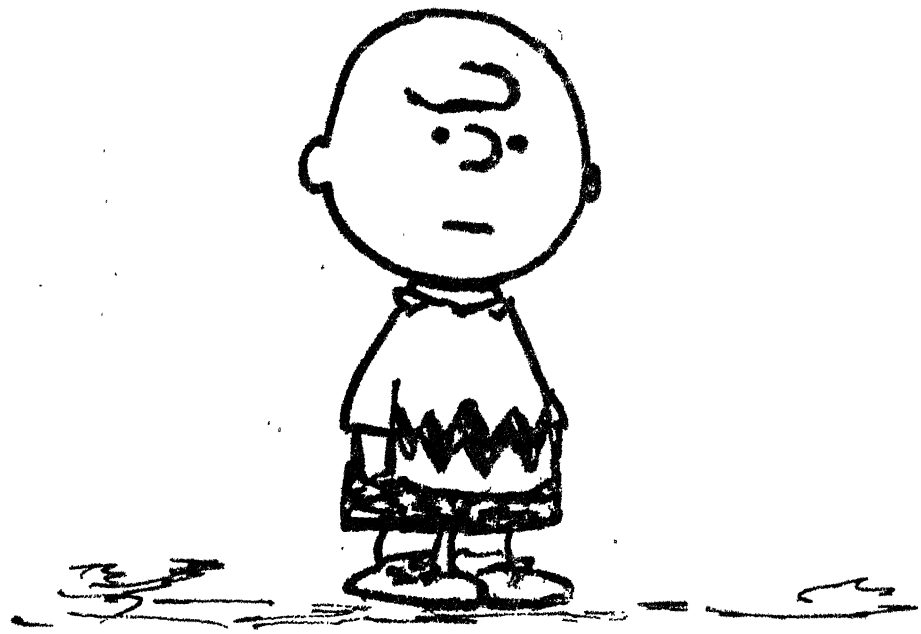
method. The chapter includes a critical comparison of the method with other methods and its possible other applications.

Chapter III describes an eclectic method for the large scale isolation and purification of three major phospholipids (phosphatidylcholine, phosphatidylethanolamine and cardiolipin) from bovine heart. This method is simpler and more economical with the solvents and requires much less solvent evaporation under reduced pressure, which was a heavy constraint because of an unpredictable and poor availability of refrigerants like liquid nitrogen or dry ice. The method is based on the experience gained with many unsuccessful attempts with the earlier methods within the limits of our resources. A critical comparison of this method with the earlier methods concludes this chapter.

Chapter IV describes a convenient and versatile method for the preparation of phospholipid vesicles of different size distributions reproducibly. In this method an alcoholic solution of the phospholipid is passed through a porous glass disc, with the help of a simple device, into a stirred aqueous medium under controllable conditions. The influence of various methodological parameters on the size distribution of the vesicle obtained as estimated by dissymmetry measurements are reported and on the basis of these observations a plausible mechanism for vesicle formation by this method is proposed. A critical appraisal of the other methods and their comparison with the present method concludes this chapter.

In Chapter V, the results of some turbidimetric studies on the cardiolipin vesicle stability vis-a-vis the calcium ion induced transformation and the effect of size and temperature on turbidity change measurements are reported. The studies although limited do indicate some interesting features, which show how this simple method may be used fruitfully to obtain some information on these transformations. It is surmised that the sigmoidal curve of Ca^{2+} vs. turbidity change rate can be interpreted in terms of the nucleation phenomenon which requires the formation of some critical clusters of vesicles before the phase transition sets in.

To



Charlie
Brown

Prologue

I am a physical chemist by training and choice. When I joined the graduate program in Chemistry, I hardly knew a word about life sciences. However, I was interested somehow to do research in Biophysical Chemistry. Dr. Gupta-Bhaya had then only recently joined about an year back and the Biosystems Lab. was in an embryonic stage.

We initially planned to do some theoretical and experimental studies in application of magnetic resonance techniques to studies on peptides. Towards this end Dr. Gupta-Bhaya advised me to equip myself well with a good mathematical and theoretical background. I, therefore, did some courses in advanced mathematics and physics. But our plans did not materialize because no working NMR instrument was readily available.

Meanwhile, I was getting interested in using our new acquisitions in the Biolabs. We, therefore, decided, somehow, to study the interaction of Ca^{2+} with mitochondrial membranes. I, therefore, fabricated a Potter-Elvejam's homogeniser and an Clark's oxygen electrode. It was an interesting experience. I started isolating mitochondria from rat liver by trapping rats in our lab., although later I used rats from animal house of CDRI Lucknow also. One day, during one of my such trips to CDRI Lucknow to get rats, I read an article in TIBS on interaction of metal ions with membranes by R.J.P. Williams and H. Hauser. I

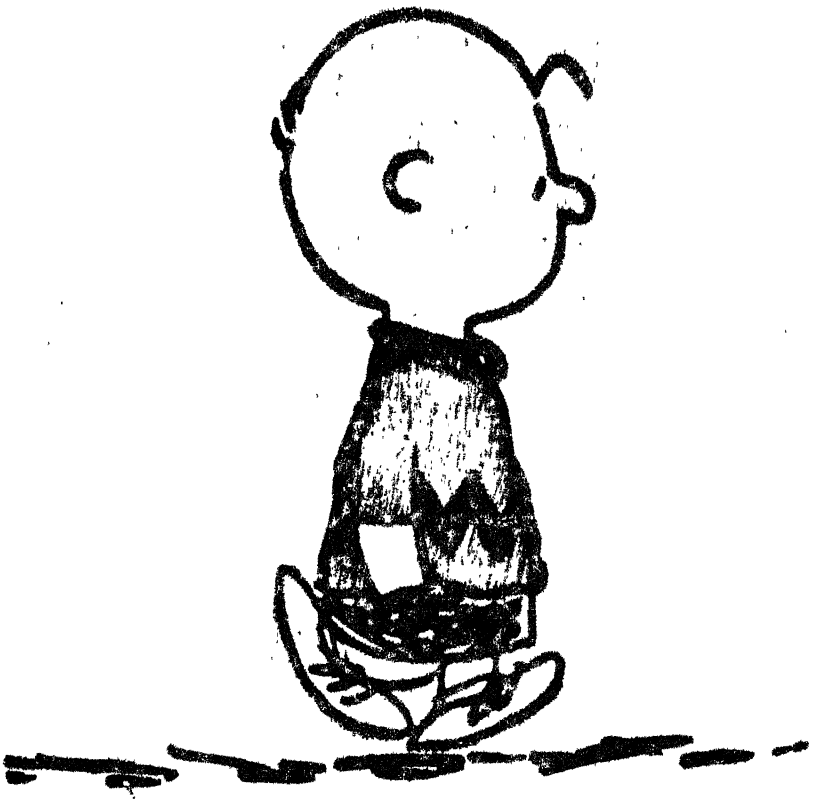
was immediately attracted by this field, because for, one thing, I was not very confident with mitochondria, as I had no guidance from any person, only the books were my guidance. Also, I felt that my instincts as a physical chemist cannot be satisfied while studying a very complex system like mitochondria. So, I wanted to work on cardiolipin the major phospholipid component of mitochondria. We, therefore, decided that we will study the interaction of Ca^{2+} with cardiolipin vesicles using micro-calorimetery, which we planned to fabricate and other techniques. For quite sometime I was involved in looking through the designs of various micro-calorimeters, to choose one which could be easily fabricated under the local conditions. We finally decided to make one on the lines of one described in Thermo-chemica Acta (1976) by Ross and Goldberg. This design appeared to be simple to fabricate, but when we enquired for the purchase of thermopiles used in their design, for a long time we received no reply. So unwillingly I had to shelve the idea. This project has since been taken over by Dr. Gupta-Bhaya and will soon be completed.

I, then, decided to do some turbidimetric studies. At least we had a working Toshniwal spectrophotometer readily available. For these studies, I found that it would be helpful if we could have a recording facility. So for some time I tried my hand in electronics trying to make a logarithmic ratio

amplifier, using FET operational amplifiers, for directly recording the absorbance. I learnt a bit of electronics in the process, but the project was not completed, because I was distracted by other more important jobs. We, then purchased a ECIL electrometer amplifier, which I interfaced between the spectrophotometer and the recorder in order to record the relative transmitted light intensity.

All the rest, follows.

PARÁDOS



Chapter I

Thermodynamically, biological cells, the basic units of living systems, are classified as far from equilibrium open systems. Survival of such systems requires boundaries which are selective in mass transport and are flexible and versatile enough to adapt to different environmental stimuli. The biomembranes which have evolved to serve these functions have invariably phospholipids as one of the major components. Physico chemical studies on phospholipid systems, therefore, constitute an important branch of molecular biology.

Phospholipids may be classified into phosphoglycerides (any derivative of sn-3-glycerophosphoric acid that contains at least one O-acyl, O-alkyl, Or O-alk-1'-enyl residue attached to the glycerol moeity), phosphoglycolipids, phosphosphingolipids, and phosphonolipids (Table 1). The phospholipid molecules are in general large enough for different parts of the molecule to be distinguishable. These molecular parts without being independent can act in autonomous ways with the neighbouring molecules. For example phosphoglycerides (Fig. 1) the most abundant form of phospholipids, are longish amphipathic molecules. At one end of these molecules there is a group with a permanent dipole moment or charge, while the rest of the molecule is made up of hydrocarbon chains. In their interaction

TABLE 1

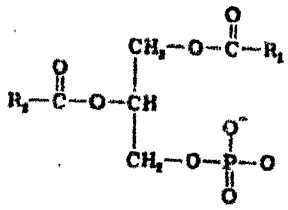
A CLASSIFICATION OF KNOWN PHOSPHOLIPIDS[a]

Classes[b]	*Analogues*[c]				
	(a)	(b)	(c)	(d)	(e)
A. Phosphoglycerides					
(*1*) Phosphatidic acids	+	+	+	+	
(*2*) Cytidylic phosphoglycerides (CDPdiglyceride)		+			
(*3*) Choline phosphoglycerides	+	+	+	+	+
(*4*) Ethanolamine phosphoglycerides	+	+	+	+	+
(*5*) *N*-Methylethanolamine phosphoglycerides		+	+		
(*6*) *N,N*-dimethylethanolamine phosphoglycerides		+			
(*7*) *N*-acyl ethanolamine phosphoglyceride		+			
(*8*) Serine phosphoglycerides		+	+		
(*9*) *N*-2-(Hydroxyethyl)alanine phosphoglyceride		+			
(*10*) Glycerol phosphoglycerides		+			+
(*11*) Glycerophosphate phosphoglycerides		+			+
(*12*) Phosphatidylglycerol phosphoglyceride (Diphosphatidylglycerol)		+			
(*13*) Mono- and diacylglycerol phosphoglycerides (lyso bis phosphatidic acids)	+	+			
(*14*) Glucosaminylglycerol phosphoglyceride					
(*15*) *O*-amino acid esters of glycerol phosphoglycerides		+			
(*16*) Inositol phosphoglyceride		+			
(*17*) Inositol monophosphate phosphoglyceride		+			
(*18*) Inositol diphosphate phosphoglyceride		+			
(*19*) Monomannosyl–hexamannosyl inositol phosphoglycerides		+			
(*20*) Glucose phosphoglyceride		+			
(*21*) *O*-diglucosylglycerol phosphoglyceride		+			
B. Phosphoglycolipids					
(*1*) Diacyl (glycerylphosphoryldiglucosyl)glycerol					
C. Phosphodiol lipids					
(*1*) Acyl dihydroxyacetone phosphate					
(*2*) Alkyl dihydroxyacetone phosphate					
D. Phosphosphingolipids					
(*1*) Sphingomyelin (Ceramide phosphorylcholine)					
(*2*) Ceramide phosphorylethanolamine					
(*3*) Ceramide phosphorylglycerol					
(*4*) Ceramide phosphorylglycerophosphate					
(*5*) Ceramide phosphorylinositol-containing lipids					
E. Phosphonolipids					

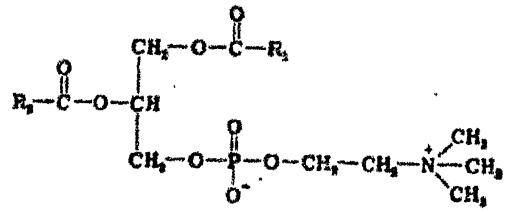
[a]An attempt has been made to conform to the recommendations of the IUPAC–IUB Commission of Nomenclature[19].

[b]The more general generic terms ('Phosphoglycerides', 'Phosphoglycolipids', 'Phosphodiol lipids' and 'Phosphosphingolipids') may be easily fitted into any general classification of lipids.

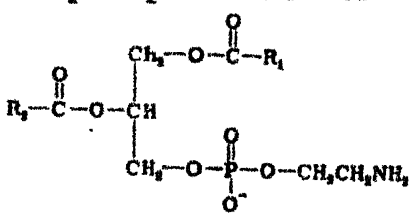
[c](a), 1-acyl-, or 2-acyl-(i.e. lyso); (b), 1,2-diacyl- (i.e. phosphatidyl); (c), 1-alk-1'-enyl-, 2-acyl- (i.e. plasmalogen); (d), 1-alkyl-, 2-acyl- and (e), 2,3-dialkyl.



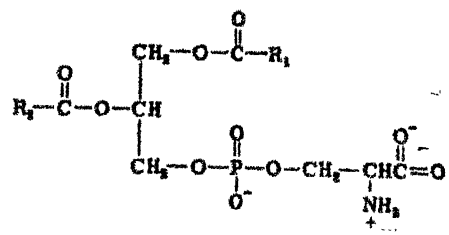
phosphatidic acid



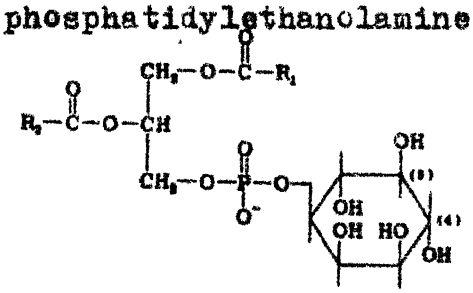
phosphatidylcholine



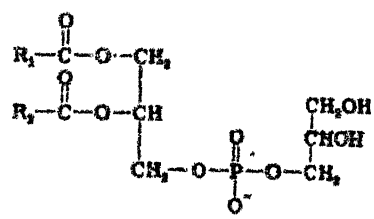
phosphatidylethanolamine



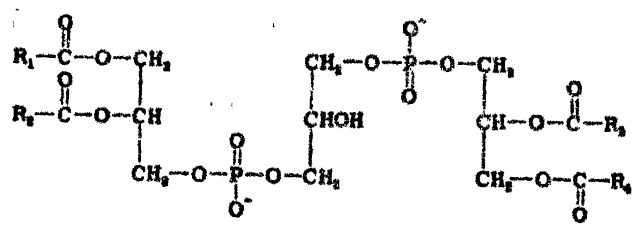
phosphatidylserine



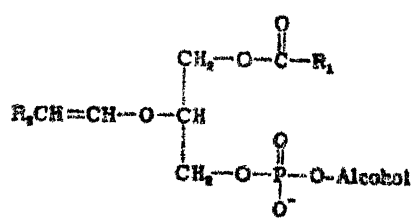
phosphatidylinositol



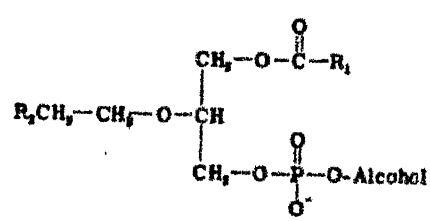
phosphatidylglycerol



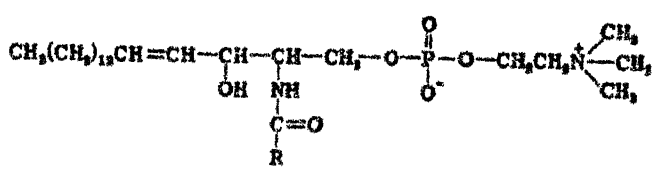
diphosphatidylglycerol(cardilipin)



plasmalogen



(saturated ether form



sphingomyelin

Fig. 1 Structures of some phosphoglycerides

with other molecules the forces that are operative at these two ends are well known to be different. As a result of the relative magnitude of these forces, phospholipids are seldom molecularly dispersed in solutions. They are present as aggregates of various sizes and shapes in solutions. At the interface of water and air or some immiscible organic solvent, the phospholipid molecules orient themselves in such a way that the paraffin chain remains in organic solvent or air, while only the polar group interact with water. This is the reason why phospholipid molecules are often referred to as having a hydrophilic head and a hydrophobic tail.

The structure and size of phospholipid aggregates in a solvent depend on the nature of the solvent, nature of the head group, the number, length and degree of unsaturation of the hydrocarbon chains and the concentration. In a particular solvent at a given concentration, the stability of any assumed aggregate can be calculated from geometric considerations, like optimal surface area (a_o), the volume of hydrocarbon chain (v), and maximum length a chain can assume (l_c). These factors can be used to determine the Gibbs free energy and hence the stability. It turns out that the magnitude of the ratio $v/a_o l_c$, called the critical packing parameter can be used to predict what structure a given system will assume (1) (Fig. 2). However, these geometric parameters are not properties of phospholipid molecules in isolation. The interactions with other

Phospholipid/Solvent	Critical packing parameter $v/a_0 l_c$	Critical packing shape	Structures formed
Short chain (C_2-C_7) diphosphatidyl lipids/water (Na^+,K^+) Some lysophospholipids (C_2-C_{16})/H_2O Long chain(C_{10}-C_{16}) diphosphatidyl lipids/CH_3OH, CH_3CH_2OH	$< \frac{1}{3}$	Cone	Spherical micelles
Lysolecithin, Long chain (C_{10}-C_{16})diphosphatidyl lipids/acetone,higher alcohols,or alcohol + water	$\frac{1}{3} - \frac{1}{2}$	Truncated cone or wedge	Globular or cylindrical micelles
Some long chain (C_{12}-C_{18}) diphosphatidyl lipids, as PC; PS; PG; CL; PA; SM/H_2O(Na^+,K^+)	$\frac{1}{2} - 1$	Truncated cone	Flexible bilayers Vesicles
Long chain (C_{12}-C_{18})diphosphatidyl lipids e.g. PS/H_2O (High Na^+,K^+) PI,PS/H_2O(Ca^{2+}, Mg^{2+})	~ 1	Cylinder	Planar bilayers
Some long chain (C_{12}-C_{18}) phospholipids, e.g., CL; PA/H_2O(Ca^{2+}, Mg^{2+}) long chain diphosphatidyl lipids/Benzene, CCl_4, $CHCl_3$, C_6H_{12} etc.	> 1	Inverted truncated cone	Inverted micelles

Fig. 2 Dynamic packing properties of some phospholipids and the structures they form in different solvents. PA = phosphatidic acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PI = phosphatidylinositol, CL = cardiolipin and SM = sphinghomyelin. Partly reproduced from (1).

molecules in the solvent influence their magnitude. Thus while acidic phospholipids like phosphatidylserine, cardiolipin etc. can form bilayered structures in aqueous systems in the absence of polyvalent metal ions at higher pH, because the repulsion of the charged head groups favor larger a_o, in the presence of polyvalent metal ions, since these repulsions are neutralised, hexagonal phase (H_{11}) is the preferred structure. In a similar vein temperature can affect l_c and v.

The size of the aggregates formed in a particular solvent can be apparently related to solubility. Shorter chain phospholipids form smaller micellar structures in polar solvents like alcohols, acetone etc. and are more soluble in these solvents than in non polar solvents like benzene, hydrocarbons etc. where they form larger reverse micellar structures. On the other hand the situation with longer chain phospholipids is just the reverse. Solubility also depends on the degree of unsaturation of the fatty acid chains. While the saturated long chain phospholipids are almost insoluble in acetone, ethyl ether and petroleum ether, their unsaturated counterparts are much more soluble in these solvents (2). Presence of one phospholipid can also markedly affect the solubility of another phospholipid in a solvent.

Phospholipids exhibit very interesting behavior in the presence of water. They are in general very hygroscopic substances. About 10-20 molecules of water per molecule of

phospholipid can be absorbed at 25°C (3-5). In general they do not pass directly from crystalline state to a solution in water. Various hydrated phases are encountered before solution of phospholipids in water occurs. Such behavior is called lyotropic mesomorphism. The lyotropic phases exhibit thermotropic mesomorphism, i.e. the particular phase obtained is a function of both water content and temperature. Phosphoglycerides and sphingolipids in bilayer systems undergo a thermotropic phase transition from gel to liquid crystalline state much below the true melting point of anhydrous solids (6-8). In the strict sense, this phase transition is not first order, as is for example the melting of ice. During this transition the gel state which is characterized by crystalline order gives way to a liquid crystalline state. In this state the crystalline order of the polar head group is retained but a degree of disorder usually associated with the liquid state arises in the hydrocarbon chain core of the bilayer. In one component systems the transition is well defined with a thermal half width of less than 1°C and enthalpy, changes between 5-10 kcal/mole of phospholipid. As is apparent from Fig. 2 acidic phospholipid bilayers can undergo an isothermal phase transition to a hexagonal phase by changes in ion content of water.

In most biomembranes, the long chain phosphatidylcholines are the major phospholipid. It is therefore, not very surprising that the structure of biomembranes as revealed by various

techniques, is composed of large areas of phospholipid bilayers. When phosphatidyl choline is dispersed in water, above its thermotropic phase transition temperature, large multi-lammellar closed sacs, commonly referred to as liposomes are formed. This dispersion is rather hetrogeneous in size and shape of liposomes. If the liposomal dispersion is irradiated with ultrasonic radiation (freq. 20-100 KHz) smaller unilamellar sacs called vesicles are formed (12). This dispersion is fairly homogeneous consisting largely of aggregates of radii 10-20 nm. A much more homogeneous dispersion can be obtained by gel filtration (13) or ultracentrifugation (14) of this dispersion. Some simple methods for detection of presence of large vesicles in small vesicle dispersions have been described recently (15,16). Other phospholipids except pure phosphatidyl ethanolamine also form vesicles under certain conditions.

In the past few years liposomes and vesicles have become increasingly popular as model biomembrane systems (17) and as possible drug carriers (18,19).

Successful application of drugs is largely dependent on selective action since the target (e.g. cells) by and large, share a number of similar attributes with normal non target areas. Therefore, unless the interaction between the target, and the drug is based on a unique property of the former, (as in antimicrobial therapy') so called side effects will occur,

as for example in chemeotherapy of cancer. An additional important problem sometimes faced is the inability to administer the drug conveniently to affected areas, as for example in the treatment of many parasitic diseases where the microorganism inside the cellular organelles are inaccessible to a wide range of otherwise effective agents. Yet in another situation administration of a drug through a particular route, which would have been beneficial to the patient is impossible because of the drug properties. Oral treatment of diabetes for example, is prevented by hormones vulnerability in the gut. Manipulation of body's defence mechanisms as in immunization against diseases is another area of medicine which would greatly benefit if the vaccines were made more effective through the use of an adjuvent aceptable to human beings. A good adjuvent could reduce the amount of antigen needed in an immunization program (resulting in savings especially relevant to developing countries like ours) and would render some vaccines more effective. Liposomes and vesicles have been implicated as possible solutions to all these problems. The wide success achieved so far, has prompted some scientists to draw the 'Trojan Horse' metaphor for liposome interaction with the cells. Just as the Greek soldiers penetrated Troy within a horse so can we introduce a wide range of materials into the cell entrapped within liposomes or vesicles. Liposomes have been, for example, found effective for oral

administration of insulin and for treatment of Kala ajar a deadly disease in tropical countries.

An effective exploitation of this drug carrier concept is possible only if we have a good understanding of the physico chemical behavior of vesicles and liposomes. This knowledge also adds to our understanding of the structure and dynamics of biomembranes. Vesicles and liposomes can be to medicine what IC's are to electronics.

The physico-chemical properties of phospholipid vesicles depend on the phospholipid composition (the nature of the head group, the fatty acid composition) the composition of the aqueous medium, (pH, ionic strength, the nature and concentration of other dissolved substances) and the size of the vesicles besides temperature and pressure.

For any physico chemical study of phospholipid vesicles a factor which can be of paramount importance is the stability of the dispersion during the investigation, phospholipids with unsaturated fatty acid chains are known to undergo auto-oxidation (20). Besides, the fatty acid ester bonds can be hydrolysed. Changes in temperature or ionic environment can cause bilayer instabilities. From a thermodynamic viewpoint, for a given phospholipid, under a particular set of enviornmental conditions, l_c and v are defined and a close interval of a_o would be permissible at equilibrium. However vesicles are

formed under far from equilibrium conditions, therefore, the size distribution of a vesicle dispersion is determined by kinetic factors and not thermodynamic. One can prepare phospholipid vesicles of a wide range of size distributions by various methods. One can break larger multilamellar liposomes into smaller lamellae which reform into smaller vesicles by extrusion, through fine pores of a membrane filter or a French press cell, besides sonication, or one can control the fusion of smaller micellar aggregates to form vesicles of different sizes. Once the vesicles are formed there are energy barriers for their close encounters necessary for fusion, or fission which can bring the system to an equilibrium distribution. Thus although vesicles of a certain size may not be thermodynamically stable they can be kinetically stable.

There are some studies of stability of sonicated phosphatidyl choline vesicles as a function of temperature and time. However, no clear consensus seems to appear from these studies. While distearoylphosphatidyl choline vesicles seem to fuse below the phase transition temperature but are stable above the phase transition temperature (21); dimyristoylphosphatidyl choline vesicles show a sharp change in size related properties above the transition temperature (22).

Size of the vesicles is an interesting parameter in their physico-chemical studies from the point of view of their stability

and also in context of their use as model biomembranes. It is well known that biomembranes exhibit regions of widely different radius of curvature. For example membranes of small neurotransmitter storage vesicles in synaptic regions of neurons, the highly convoluted cristea of mitochondrial inner membrane, and the brush borders of intestinal epithelial cells have regions of very small radius of curvature (about 75 A). It is possible that curvature provides a means of regional differentiation of membrane function.

The functions of biomembranes depend on their phospholipid composition. Thus phosphatidyl serine is found in higher proportions in brain and associated organs, while cardiolipin is concentrated mainly in those organs which are richer in mitochondria. In mitochondria, cardiolipin is concentrated in the inner membrane which is the site of oxidative phosphorylation. Some enzymes like mitochondrial ATPase exhibit specific affinity for cardiolipin. The functions of neurons and mitochondria are controlled by Ca^{2+} ions, and acidic phospholipids can undergo phase transitions induced by these ions. Thus one can see that the phospholipid bilayers are not just passive semipermeable boundaries but rather play an active role in cellular processes (23,24).

In the light of the above discussion it is not surprising that a very wide cross-section of scientists from various disciplines are increasingly getting interested in studies on phospholipid vesicles. A vast array of methodologies has been

developed in the past few years (25). To begin with there has been a considerable advance in the methods for isolation and purification of phospholipids from biomaterials (26). Many different schemes for the synthesis of phospholipids have been elucidated (27). One can now synthesize or purchase many phospholipids with different fatty acid compositions. This has added a new dimension to the physico-chemical studies, since the results with synthetic phospholipids are much more amenable to quantitative analysis than those with isolated phospholipids which are invariably a complex mixture of many fatty acid esters. Also one can synthesize labelled phospholipids for various spectroscopic studies. One can now study the effect of various molecular parameters on different physico-chemical properties. The structure elucidation of phospholipids has also become easier with the wide spread use of more sophisticated physical methods (28).

In the following few paragraphs the methodologies and results of some physico-chemical studies on permeability, thermotropic phase transition, effect of ion environment and effect of size of the phospholipid vesicles on some molecular properties in bilayers are briefly discussed.

Permeability is one of the most widely studied property of phospholipid bilayers. Several methods have been developed to study the permeability of vesicles. The substance (a radioactive tracer or otherwise) is added to the salt solution during

the formation of the vesicles, when it is trapped in the aqueous core of the closed sacs. The vesicles with the trapped substance are then separated from the untrapped substance by dialysis or gel filtration. The efflux of the trapped substance can then be monitored by sequential dialysis of vesicle solution against the same salt solution by radiochemical photo-chemical or electro-chemical methods (29-31).

One of the most striking characteristics of unmodified phospholipid membranes is their impermeability to cations. The permeability coefficients vary from 10^{-13}-10^{-14} cm/sec. for monovalent cations. Most biomembranes show a discrimination in Na^+/K^+ transport. However, only acidic phospholipids show any such discrimination in pure phospholipid vesicles. The ionic permeability for cations can be increased manyfold and specifically by a number of ionophoric molecules. These ionophores have polar interior, where the cation is bound and a nonpolar exterior which easily dissolves in the bilayer core. The diffusion coefficients for anions and neutral molecules are sufficiently higher (diffusion coefficient : 10^{-6}-10^{-10} cm/sec.).

The permeability of a bilayer can be related to its fluidity, which is determined by the packing density of the phospholipid molecules. Larger and smaller vasicles are expected to have different packing density and it has been observed on the basis of some kinetic studies of water diffusion that smaller vesicles have looser packing (32). Divalent metal ions which can also

affect the packing of phospholipid molecules in bilayers are known to significantly increase the permeability (10).

Thermotropic phase transitions of phospholipids have been studied by a wide variety of physical methods. These include differential scanning calorimetry, spin probes, NMR, IR, Raman, and fluorescence spectroscopy, dialatometry and turbidimetry. Two phospholipids which have been much studied in bilayer systems are dimyristoyl and dipalmatoyl phosphatidylcholines. These two saturated acyl chain phospholipids have transition temperatures of 24.4 and 41.1°C respectively. Both transitions are preceded by a so called pretransition which is associated with a comparatively small enthalpic change. The origin of this pretransition is currently a subject of considerable interest (33). In small single bilayer vesicles the characteristic temperature of transition of these two phospholipids is lowered by 3.5 and 4.7°C respectively. In addition, the width of the transition is increased. This reflects on the cooperativity of the transition. The pretransition is absent in vesicles of minimum radius of curvature (33). Introduction of one cis double bond into either of these two saturated phosphatidylcholines lowers the transition temperature well below 0°C. Thus most naturally occurring phosphatidylcholines which have at least one unsaturated acyl chain in 2 position of glycerol backbone, have phase transition temperature below 0°C.

The thermotropic phase transition characteristics of some dipalmatoyl phosphoglycerides are summarized in Table 2. In general the phase transition characteristics of multi-component bilayers is complex. In two component systems, the behaviour generally fall within the range defined by following extremes. If the component lipids are similar in generic type eg. phosphatidyl cholines differing only in acyl chain length by two methylene groups, gel formation does not alter the distribution of the components. In both the gel and the liquid crystalline phase the two components are miscible in all proportions. This type of system behaves as an ideal mixture with the values of the transition parameters given by a mole fraction weighted average of the corresponding parameters of the pure components. A good example of this type of behavior is liposomes comprised of DMPC and DPPC (39). At the other extreme are systems comprised of dissimilar type of lipids with phase transition temperatures differing by at least 10°C. In this case phase separation may occur with liquid crystalline phases consisting predominantly of one component. In systems of this type, the coexistence of gel and liquid crystalline phases of very different composition may occur over a large composition and temperature range (39).

The various molecular changes that occur at the transition temperature in phosphatidylcholine bilayers may be summarized as follows :

a) an expansion of lattice and decrease in bilayer thickness.

Table 2

Thermodynamic data for crystalline to liquid crystalline transition of 1,2 - Dipalmitoyl phospholipids at maximum hydration (pH ~ 7)

Phospholipid	Net charge	T_c °C	ΔH KCal/ mole	Ref.
Phosphatidycholine	0	41	8.7	(34)
Phosphatidylglycerol*	-1	41	7.9	(35)
Phosphatidylserine*	-1	55	3	(36)
Phosphatidylethanolamine	0	63	8.1	(37)
Phosphatidic acid*	-1	67	5.2	(35)
Diphosphatidylglycerol*	-2	39.7	8.9	(38)

*The transition of these phospholipids is very sensitive to the composition of the aqueous phase.

b) increased rotational isomerisation of CH_2 groups about C-C bond

c) increased mobility of the $-N(CH_3)_3$ groups

d) increased diffusion rate of lipids above the transition temperature

e) some changes in bound water interaction at the transition temperature.

We also know that the thermotropic transitions can be shifted by interaction with metal ions, pH, polypeptide or protein interactions. Furthermore, the permeability characteristics for various molecules, the filtration characteristics through membrane filters (40), and aggregration characteristics (41) are also dependent upon whether the lipid is above or below its transition temperature.

From the structural viewpoint, below the transition temperature, the hydrocarbon chains are in a relatively rigid all trans conformation. As the temperature is raised to the region of transition temperature, the hydrocarbon chains are disordered by undergoing rapid trans-gauch rotational isomerizations along the chains, but the hydrocarbon chains maintain an average orientation perpendicular to the plane of the bilayer. Direct observation of an increase in gauch-trans ratio of the saturated phospholipids has been demonstrated by vibrational Raman spectra (42). Accompanying the endothermic transition the trans-gauch

isomerizations of the hydrocarbon chains can be interpreted by a 2g1 kink formation (43). The kink model provides a qualitative description of the lateral expansion and decrease in bilayer thickness.

Effect of Ionic Environment : Phosphatidylcholine and phosphatidylethanolamine are isoelectric over a wide range of pH. This has been demonstrated by studying the pH dependence of a number of physico-chemical properties. The electrophoretic mobility (zeta potential) of phosphatidylcholine vesicles remains unchanged between pH 3-11 (44) as does the chemical shifts in egg phosphatidylcholine (45). Phosphatidylethanolamine behaves differently in so far that the primary ammonium group becomes deprotonated at pH 8. The physico-chemical behavior of acidic phospholipids is strongly pH dependent. The dissociation constants for some phosphoglycerides are presented in Table 3.

Phospholipid bilayers presumably supply binding sites for many ions necessary for various biochemical processes like nerve excitation, ion translocation and enzyme activity. However, although it has long since been known that acidic phospholipids have a high affinity for divalent cations (46) many molecular details of the interaction are still obscure. The interaction of the phospholipid with metal ions has been studied both with monolayers and bilayers. In studies with monolayers, the binding is monitored by the use of radioactive

Table 3

Apparent pK values of some phospholipids

Lipid	pK		Solvent
	Ionic group	Ionic group	
Phosphatidyl-ethanolamine	$-PO_4$ 4.1	$-NH_2$ 7.8	H_2O
Phosphatidic acid	$-PO_4H_2$ 3.8 3.0	$-PO_4H$ 8.6 8.0	H_2O 0.1M NaCl or KCl
Phosphatidylionistol	PO_4H 3.12 2.50		H_2O 0.08M HCl
Phosphatidylserine	-COOH 4.2 3.0	$-NH_2$ 9.4 10.0	H_2O 0.2M KCl

Table 4

Some Binding Data of Ca^{2+} binding to Acidic phospholipids

Phospholipid	Conditions	Ca_T	K	Ref.
Phosphatidic acid	.03M NaCl, pH 7.23, .05M Tris, 24°C	5.9×10^{-5} 1.39×10^{-4}	$.85 \times 10^{4}$ 1.33×10^{4}	(58)
Phosphatidylserine	Monolayers	10^{-7} 10^{-3}	10^{6} 10^{4}	(54)

isotopes. The method is based on the detection of soft (short range) radiations emanating from the air water interface. The radiation from the bulk phase do not reach the detector because it gets quenched (47). The surface radioactivity measurements yield the amount of metal ion bound and hence the apparent binding constant. This method is not very sensitive and hence there was an early confusion about the binding of Ca^{2+} to phosphatidylcholine monolayers. While the surface radioactivity measurements showed almost no interaction (48), the surface potential measurements showed clear changes (49). This confusion has since been cleared, using lanthanide ions as isomorphous replacement for Ca^{2+} (45). The stoichiometry of metal ion/lipid molar ratio for interaction of lanthanide ions or Ca^{2+} with phosphatidylcholine depends on the experimental conditions. In anhydrous methanol, where the lipid is known to form smaller micellar aggregates the ratio is reported to be one for interaction Ca^{2+}, Mg^{2+} and Ce^{3+} (50). However, 2:1 complexes are formed with the phosphatidylcholine bilayers present in water as unilamellar vesicles (49). It has been shown that there is only one binding site per lipid molecule from changes in chemical shifts and broadening probes of lanthanide series (51). The observed shift of ^{31}P resonance contains both the contact and the pseudocontact terms while the ^{1}H shifts of CH_2O groups next to the phosphate groups are mainly pseudo contact in origin. This and the changes in

the linewidth in the presence of Gd^{3+} indicate that the phosphodiester group is the only binding site. It has also been shown that in the phospholipid bilayers the metal ion is coordinated to two oxygen atoms of phosphodiester groups of two neighbouring lipid molecules (52). The equilibrium constants determined from ^{1}H and ^{31}P chemical shifts seem to depend on the loading i.e., the amount of metal ion bound to the lipid surface, the ionic strength and the nature of anion added but is independent of the pH (51). Assuming a 1:2 complex the equilibrium constant is about $10^{4}L^{2}M^{-2}$ at 0.15 M KCl and $3x10^{3}L^{2}M^{-2}$ at zero KCl concentration.

The fact that the shift induced by lanthanides depends on the nature of anions suggests that there is an association of the anion with the positively charged $-N(CH_3)_3$ group. The interaction is weak as is evident from the shift changes of $-^{+}N(CH_3)_3$ proton resonance induced by halides (53). The interaction of the lanthanides is generally enhanced in the presence of anions. The order of enhancement is $Cl^{-} < Br^{-} < NO_3^{-} < SCN^{-} < I^{-} < ClO_4^{-}$ (51). This is also the order of effectiveness of these anions in reducing the positive zeta potential of phosphatidylcholine bilayers to which lanthanides are bound. The conformation of the head group bound to the lanthanides is more extended in bilayers of larger radius of curvature (54).

As expected acidic phospholipids interact with metal ions much more strongly because of net negative charge on the head group at neutral pH. The negative charge gives rise to a repulsion between neighboring phospholipid molecules in a monolayer or a bilayer, as well as a net repulsion between the bilayers of different liposomes (55). Addition of metal ions leads to charge neutralization and the condensation of monolayers as revealed by changes in surface pressure (56). The binding of metal ions to acidic phospholipids have been investigated using a number of techniques like titrimetry (57) turbidimetry (58), NMR (59) and surface radioactivity (60-62) measurements. Some data on the binding constants is summarized in Table 3. As may be expected in the presence of different metal ions the binding is competitive and the polyvalent metal ions bind much more strongly then the monovalent metal ion concentration, This may be explained by a decrease in surface potential (ψ) and the observed binding constants K_a may be related to the intrinsic binding constant K_i by the relation

$$K_a = K_i \exp(-ze\psi_o/kT)$$

where k is the Boltzman constant and T the absolute temperature. The binding sites cannot be decided unambiguously in all cases, especially in case of phosphatidylserine (60).

The neutralization of charge on addition of some cations leads to the aggregation of vesicles. Monovalent ions are known to induce aggregation of acidic phospholipid vesicles (62). Polyvalent metal ions, since they neutralize the charge more effectively induce aggregration at much lower concentrations (65). In the presence of higher concentrations of polyvalent metal ions the bilayer to hexagonal phase transition occurs. This changes the binding behavior also. Thus as the total Ca^{2+} concentration is increased from 0.2 to 1 mM there is a sharp change in the mole ratio of the bound Ca^{2+} to phosphatidylserine from 0.12 to 0.5 (66). Beyond 1 mM the bound Ca^{2+} reaches a plateau. The Hill plot shows some cooperativity. Freeze fracture electron microscopic photographs show that in the hexagonal phase phosphatidylserine forms cochelate type structures. Mg^{2+} does not cause fusion and phase transitions in phosphatidylserine vesicles even at ten times the concentration of Ca^{2+} required to do so (66). This indicates a difference in the binding behavior of Ca^{2+} and Mg^{2+} to phosphatidylserine bilayers (57). The phase transition is accompanied by the crystallization of the acyl chains and the thermotropic phase transition temperature is shifted to higher temperatures. ^{31}P NMR linewidth confirms this crystallization. For phosphatidylserine no thermotropic phase transition takes place upto 80°C while Ca^{2+} - palmatoyl cardiolipin exhibits phase transition at 81.3°C(38). It has been

proposed (66) that all the Ca^{2+} binding sites must be occupied in order to trigger the phase transition. The binding of Ca^{2+} to the outer monolayer of the vesicles causes a transient instability which triggers the fusion and phase transition.

In mixed lipid vesicles, Ca^{2+} is known to induce phase seperation (68,69,70). In phosphatidylcholine-phosphatidylserine mixed bilayer membranes (with spin labelled PC) the broadening of esr line on addition of Ca^{2+} has been shown to indicate phase separation. The phenomenon depends on Ca^{2+} concentration very sharply. The line width sharply increases above a threshold Ca^{2+} concentration and then reaches a plateau.

The kinetics of Ca^{2+} induced aggregration of phospholidic acid-phosphatidylcholine vesicles has been studied by stopped flow turbidimetry (71). The amplitude of the scattered light increases very abruptly to its maximal value in the Ca^{2+} concentration range 1-6 mM. The rate of aggregration as measured by $1/t_{1/2}$ increases also, but above the threshold it shows only a small variation over a 100 fold range of Ca^{2+} concentration.

There has been a recent spurt of interest in Ca^{2+} induced phase changes in cardiolipin vesicles. It is known that Ca^{2+}-cardiolipin complex forms a hexagonal phase from X-ray diffraction data (9). Recently ^{31}P NMR (72) and freeze fracture electron microscopy (73) have been used to investigate the phase

transition process from bilayers in vesicles to the hexagonal (H_{11}) phase. The ^{31}P NMR studies indicate that this phase change proceeds via an intermediate phase characterized by isotropic motional averaging. Freeze fracture electron microscopy reveals aggregration and fusion which precede the phase transition and the reversal of the phase transition on addition of EDTA.

Size of the vesicles determines the radius of curvature of the bilayer. Till very recently there was no method to prepare phospholipid vesicles of different sizes. The vesicles formed by sonication are small. The size and nature (unilamellar or multilamellar) of phospholipid vesicles can be determined by electron microscopy (negative staining (74) or freeze fracture (75)) and by light scattering measurements (76,77). In the absence of any method for preparation of vesicles of different radius, some scientists have studied small sonicated vesicles and large multilamellar liposomes to study the effect of radius of curvature on various molecular properties of phospholipids in bilayers. It has been found that there is a significant dependance of the rate of internal flexibity on the bilayer curvature (32,78). This has been attributed to the differences in molecular packing in bilayers with different radius of curvature. The possibility that the observed differences may be due to different pressures maintained across bilayers of different radii has been ruled out because the NMR

spectra of lecithin vesicles do not alter under conditions which would generate much higher osmotic pressure differences. Geometric considerations can be used to show that the methyl ends of the acyl chains of the phospholipid molecules on the inner monolayer of a 300 Å dia. vesicle, occupy an area 1.5 times that covered by the charged group, whereas the opposite is true for the outer monolayer (32). This difference in packing is also reflected in the thickness of the two monolayers of a phospholipid vesicle. NMR and hydrodynamic studies show that the outer monolayer is thicker than the inner monolayer (79,80). During the thermotropic phase transition, NMR studies indicate that, while there is a change in the organization of the inner monolayer head groups, there is relatively no change for the outer monolayer (81).

The differences in the packing of phospholipid molecules in vesicles of different radii may influence their metal ion induced phase transitions also. Towards this end we thought it is timely to study the Ca^{2+} induced phase transitions in cardiolipin vesicles of different sizes, at different temperatures and vesicle concentrations.

References

1. Israelachvili, J.N., Marcelja, S. and Horn, R.G. (1980)(r) Physical principles of membrane organization, Quart. Revs. Biophys. 13, 121.

2. Dervichian, D.G. (1964)(r) The physical chemistry of phospholipids Prog. Biophys. Mol. Biol. 14, 263.

3. Elworthy, P.H. (1961) J. Chem. Soc. 815, 5385.

4. Veksli, Z., Salsbury, N.J. and Chapman, D. (1969) Biochim. Biophys. Acta, 183, 434.

5. Katz, Y. and Diamond, J.M. (1974) J. Memb. Biol. 17, 87.

6. Chapman, D. (1975) (r) Phase transitions and fluidity characteristics of lipids and cell membranes, Quart. Revs. Biophys. 8, 185.

7. Lee, A.G. (1977) (r) Lipid phase transitions and phase diagrams, Biochim. Biophys. Acta, 472, 237.

8. Melchoir, D.L. and Steim, J.M. (1979) Lipid associated thermal events in biomembranes Prog. Surf. Memb. Sci. 13, 211.

9. Rand, R.P. and Sengupta, S. (1972) Biochim. Biophys. Acta, 457, 109.

10. Papahadjopolous, D., Jacobson, K. and Poste, G. (1976), Biochim. Biophys. Acta, 448, 265.

11. Papahadjopolous, D., Jacobson, K., Poste, G. and Shepherd, D.(1975) Biochim. Biophys. Acta, 394, 483.

12. Bangham, A.D., Hill, M.W. and Miller, N.C.A. (1974) (r) Preparation and use of liposomes as models of biological membranes. Meth. Memb. Biol. 1, 1.

13. Huang, C., (1969) Biochemistry, 8, 344.

14. Barenholz, Y., Gibbs, D., Litman, B.J., Goll, J., Thompson, T.E. and Carlson, F.D. (1977) Biochemistry, 16, 2806.

15. Barrow, D.A. and Lentz, B.R. (1980) Biochim. Biophys. Acta, 597, 92.

16. Ranswoude, A.J.B.M.V., Blumenthal, R. and Weinstein, J.N. (1980), Biochim. Biophys. Acta, 575, 151.

17. Papahadjopolous, D. and Kimelberg, H.K. (1974) (r) Phospho-lipid vesicles (liposomes) as models for biological membranes their properties and interactions with proteins and cholesterol, Prog. Surf. Sci.4, 141.

18. Gregoriadis, G. (1978) (r) Liposomes in therapeutic and preventive medicine : the development of drug carrier concept Ann. New York, Acad. Sci. 308, 343.

19. Duwe, C.D., Trouet, A., Compemeere, D., and Baurein, R (1978) ibid, 226.

20. Klein, R.A. (1970) Biochim. Biophys. Acta, 210, 480.

21. Larabee, A.L. (1979) Biochemistry, 18, 3321.

22. Martin, F.J. and MacDonald, R.C. (1976), Biochemistry, 15, 321.

23. Cullis, P.R. and Kruijff, B. (1979) (r) Lipid polymorphism and the functional roles of lipids in biological membranes. Biochim. Biphys. Acta, 559, 399.

24. See for example Form and Function of Phospholipids (1973) Ansell, G.B., Hawthorne, J.N. and Dawson, R.M.C. (eds.) Elsevier Scientific Publishing Company,

25. See for example "Methods in Membrane Biology", Vols.1-10, Korn, E.D. (eds) Plenum Press.

26. See for example Spanner, S. (1973) Separation and analysis of phospholipids in (24) also Zahler, P. and Niggli, V. (1977) (r). The use of Organic solvents in membrane Biology, Vol. 8 in (25).

27. Kates, M. (1977) (r) Synthesis of stereoisomeric phospholipids for use in membrane studies ibid, 8, 219.

28. Klein, R.A. and Kemp, P. (1977) (r) Recent methods in elucidation of lipid structure ibid, 51.

29. Papahadjopolous, D., Nir, S. and Ohki, S. (1972) Biochim. Biophys. Acta, 266, 561.

30. DeGier, J., Mandershoot, J.G. and VanDeenen, L.L.M., (1968), Biochim. Biophys. Acta., 150, 666.

31. Hinkle, P.C. (1970) Biochem. Biophys. Res. Comm., 141, 1375.

32. Sheetz, M.P. and Chan, S.I. (1972) Biochemistry, 11, 4573.

33. Suurkaus, K.J., Lentz, B., Barenholz, Y. Biltonen, R.L. and Thompson, T.E. (1976) Biochemistry, 15, 1393.

34. Chapman, D., William, R.M. and Lardbrook, B.D. (1967) Chem. Phys. Lipids, 1, 445.

35. Jacobson, K. and Papahadjopolous, D. (1975) Biochemistry, 14, 152.

36. McDonald, R.C., Simon, S.A. and Baer, E. (1976) Biochemistry 15, 885.

37. Blume, A. and Ackerman, T. (1974) FEBS Letts. 43, 71.

38. Rainer, S., Jain, M.K., Ramirez, F., Ioannou, P.V., Marecek, J.F. and Weger, R. (1979) Biochim. Biophys. Acta, 558, 187.

39. Lentz, B.R., Barenholz, Y. and Thompson, T,E. (1976) Biochemistry, 15, 4521.

40. Brendzel, A.M. and Miller, I.F. (1980) Biochim. Biophys. Acta, 596, 129.

41. Peterson, N.O. and Chan, S.I. (1978) Biochim. Biophys. Acta, 509, 111.

42. Lippert, J.L. and Peticolas, W.L. (1971) Proc. Natl. Acad. Sci. U.S.A., 68, 1572.

43. Marcelija, S. (1974) Biochim. Biophys. Acta , 367, 165.

44. Bangham, A.D. and Dawson, R.M.C. (1959) Biochem. J. 72,486.

45. Hauser, H. Phillips M.C., Levine B.A. and Williams,R.J.P. (1975) Eur. J. Biochem., 58, 133.

46. Christenen, H.N. and Hasting, A.B. (1940) J. Biol. Chem., 136, 387.

47. Muramatsu, M. (1973) Surf. Coll. Sci., 6, 101.

48. Shah, D.O. and Shulman, J.H. (1965) J. Lip. Res.,8, 227.

49. Seimiya, T. and Ohki, S. (1972) Nature, New Biol., 239,26.

50. Misiorowski, R.L. and Wells, M.A. (1973) Biochemistry, 12, 967.

51. Hauser, H., Hinckley, C.C., Krebs, J., Levine, B.A., Phillips, M.C. and Williams, R.J.P. (1977) Biochim. Biophys. Acta, 468, 364.

52. Hauser, H., Phillips, M.C., Levine, B.A. and Williams, R.J.P. (1976) Nature, 261, 390.

53. Jandrasiak, G.L. (1972) Chem. Phys. Lipids, 9, 133.

54. Hauser, H., Darke, A., and Phillips, M.C. (1976) Eur. J. Biochem.62, 335.

55. Nir, S. and Bentz, J. (1978) J. Coll. Int. Sci., 65, 399.

56. Shah, D.O. and Shulman, J.H. (1965) J. Lip. Res.,6,341.

57. Henderickson, H.S. and Fullington, J.G. (1965) Biochemistry, 4, 1599.

58. Abramson, M.B., Katzman, R. and Curci, R. (1965) J. Coll. Sci., 20, 777.

60. Hauser, H., Darke, A. and Phillips, M.C. (1976) Eur. J. Biochem., 62, 344.

61. Rojas, E., and Tobias, J.M. (1965) Biochim. Biophys. Acta, 94, 394.

62. Hauser, H., Dawson, R.M.C. (1967) Eur. J. Biochem., 1, 61.

63. Nir, S. and Bentz, J., (1980) Proc. Natl. Acad. Sci. 77,4167.

64. Atkinson, D., Hauser, H., Shipley, G.G. and Stubbs, J.M. (1974), Biochim. Biophys. Acta , 339, 10.

65. Papahadjopolous, D., Vail, W.J., Jacobson, K., and Poste, G. (1975), Biochim. Biophys. Acta , 394, 483.

66. Papahadjopolous, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G., and Lazo, R. (1977) Biochim. Biophys. Acta , 465, 579.

67. Newton, C., Pangborn, W., Nir, S., and Papahadjopolous, D. (1978), Biochim. Biophys. Acta, 506, 281.

68. Ohnishi, S.I. and Ito, T. (1973) Biochem, Biophys. Res. Comm. 51, 132.

69. Ohnishi, S.I. and Ito, T. (1974) Biochemistry, 13,881.

70. Papahadjopolous, D., Poste, G., Schaeffer, B.E. and Vail, W.J. (1974), Biochim. Biophys. Acta, 311, 330.

71. Lansman, J. and Haynes, D.A. (1975) Biochim. Biophys. Acta , 394, 335.

72. Cullis, P.R., Verkleij, D.J., and Vervegaert, P.H.J.T. (1978) Biochim. Biophys. Acta, 513, 11.

73. Vail, W.J. and Stollery, J.G. (1979) Biochim. Biophys. Acta, 551, 74.

74. Glauert, A.M. and Lucy, J.A. (1969) J. Microscopy,89,1.

75. Deamer, D.W., Leonard, R., Tardieu, A. and Branton, D. (1970) Biochim. Biophys. Acta, 219, 47.

76. Selser, J.C., Yeh, Y. and Baskin, R.J. (1976), Biophys. J., 16, 377.

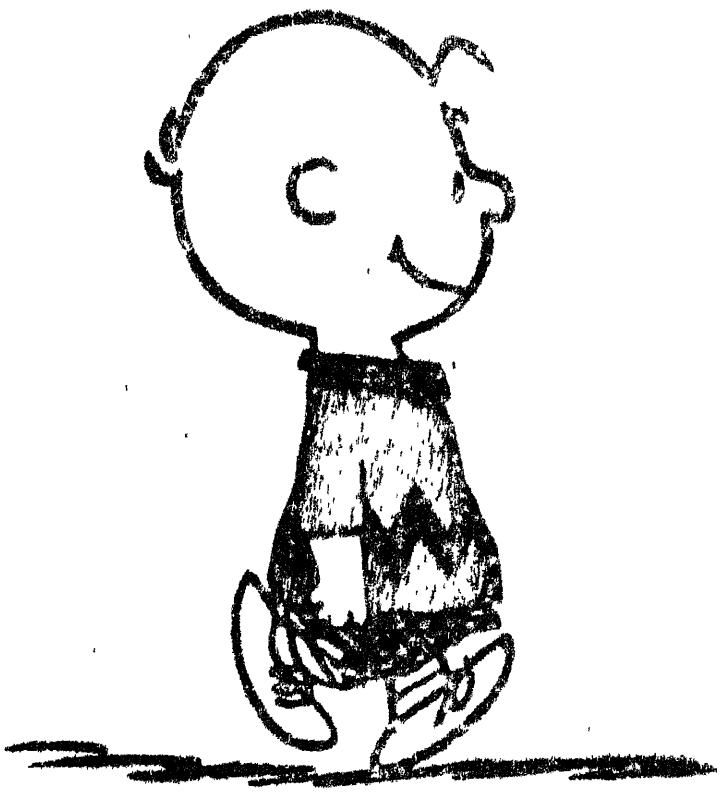
77. Chong, C.S. and Colbow, K. (1976) Biochim. Biophys. Acta, 436, 260.

78. Lichtenberg, D., Peterson, N.O., Girardet, J., Kainosho, M., Kroon, P.A. Seiter, C.H.A., Feigenson,G.W. and Chan, S.I. (1975) Biochim. Biophys. Acta, 382, 10.

79. Chrzeszcysk, A., Wishia, A. and Springer, C.S. (1977) Biochim. Biophys. Acta, 470, 161.

80. Huang, C. and Mason, J.T. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 309.

81. Eigenberg, K.E. and Chan, S.I. (1980) Biochim. Biophys. Acta, 599, 330.



Chapter II

Concentration is an important variable in physicochemical studies of solutions. Accurate determination of concentration, in cases where a standard solution cannot be prepared by dissolving in a known volume a weighed quantity of solute, therefore deserves special attention. Most of the methods used for preparation of phospholipid vesicles yield a hetrogeneous population of sizes. In order to perform a physicochemical study with vesicles of a definite size one can separate vesicles of desired size by ultra centrifugation, gel filtration or membrane filtration. However, each of these methods result in the loss of an indefinite fraction of the lipid originally dispersed. One therefore needs a method to determine the concentration of phospholipids in aqueous dispersions.

In the method most often used, the aqueous dispersion of the phospholipids is evaporated to dryness, and the lipid is then digested with concentrated perchloric acid (72%). The concentration of the phosphate ions librated is determined by reduction of phosphomolybdate complex using a variety of reagents (1-4). This method is time consuming. A typical estimation may take 3-4 hours. In addition it is not specific. If phosphate ions or other phosphate esters are present in the dispersion they will also form the complex and interfere with the determination of phospholipid concentration.

London and Feigenson (5) have reported a fluorimetric assay of phospholipids in aqueous dispersions based on fluorescence enhancement of diphenylhexatriene. However fluorescence enhancement is a function of temperature and concentration of different solutes present in the dispersion. This method, therefore, can not be used without prior standardization for each set of conditions. Raheja et al. (6) have described a method for determination of phospholipid concentration in chloroform solutions using a molybdenum reagent. In this method a lipid-molybdenum blue complex is formed, which is quantified spectrophotometrically. In order to adapt this method for aqueous dispersions one may either evaporate the dispersion to dryness or extract the phospholipid into chloroform. Evaporation to dryness unless carried out at higher temperatures is time consuming. Evaporation at higher temperatures may affect the sensitivity of the method by hydrolysis of the phospholipid. Prior extraction into chloroform has the difficulty that it needs a lot of care for complete extraction.

It was therefore felt necessary to develop a method for determination of phospholipid concentration in aqueous dispersions which (a) is specific for phospholipids; (b) does not require prior evaporation or extraction. The method of Raheja et al. (6) showed some promise as a starting point

because, this reagent is similar to the Dettmer and Lester's reagent(widely used to detect phospholipids on TLC plates) known to be specific. Addition of their reagent to an aqueous dispersion of phospholipid, followed by heating and subsequent extraction with some chloroform leads to the development of a very faint blue color in the chloroform phase. Apparently the dilution of the reagent with the aqueous dispersion has reduced the sensitivity - with a more concentrated reagent the sensitivity may be increased. An adhoc reagent in which the concentration of all the components is increased about five fold was, therefore, prepared as follows :

5 gms of ammonium molybdate was dissolved in 200 ml. of 75% sulfuric acid (solution A). 3 gms of ammonium molybdate was dissolved in 50 ml of concentrated hydrochloric acid and was then reduced by stirring with 2 ml. of mercury for about 30 minutes. The solution was then filtered and added slowly to solution A. The dark aquamarine colored solution was allowed to cool and stored in a glass stoppered bottle.

Preliminary experiments with this reagent showed promise. Therefore a systematic investigation to obtain optimal sensitivity was undertaken. It was felt that the time for heating and the volume of reagent used needs standardization. If the solution is heated for insufficient time, the complex formation may be incomplete. A small difference in time of heating may

then introduce considerable error in the final estimation. The volume of reagent used determines the concentration of complex for a given concentration of lipid. Thus sensitivity is expected to be a function of the relative concentrations of the reagent and the lipid. In addition while the reagent may be specific on TLC plates, the formation and distribution of the complex may depend on the presence of other solutes. These investigations were carried out using the following experimental protocol.

Aqueous dispersion of phosphatidylcholine (obtained from CSIR center for Biochemicals,Delhi) was prepared either by sonication or dilution of an ethanolic solution. To 2 ml of phospholipid dispersion in a glass stoppered test tube the desired volume of reagent was added. The test tube was heated in a boiling water bath for desired time and the solution was then allowed to cool for about 5 min. 3.5 ml of chloroform was then added and the two phases mixed thoroughly. The phases were allowed to separate and the absorbance of the chloroform phase was read against a blank in 10 mm cuvette on a UV Vis Spectrophotometer.

The results of these investigations are as follows :

(i) the complex has a broad absorbance maxima around 710 nm. There is no detectable absorbance of a blank at this wavelength. Therefore chloroform can be used as a blank.

(ii) Emulsions invariably form which do not clear on standing if one uses pure chloroform for extraction. Use of 9:1 chloroform - methanol helps considerably in obtaining clearer lower phase.

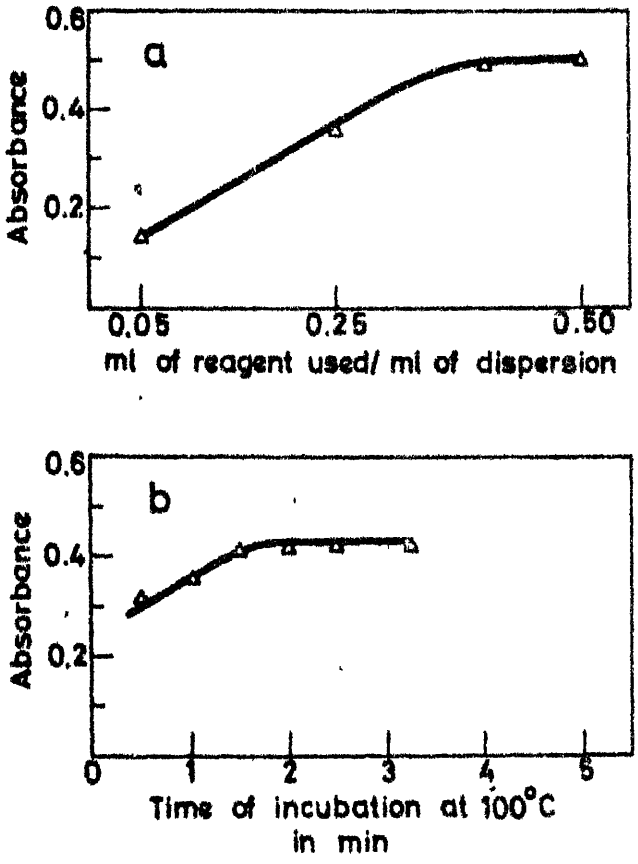
(iii) The minimum volume of reagent required to give maximum absorbance after heating for 2 mins. in a boiling water bath is 0.4 ml. /ml of lipid dispersion containing about 0.5 μmole/ml of phosphatidylcholine (Fig. 1a).

(iv) The minimum time for incubation of a mixture of 1.0 ml of reagent and 2 ml. of dispersion in a boiling water bath to obtain maximum absorbance is about 90 secs (Fig. 1b).

On the basis of these results the following method for estimation was employed for further investigations.

---

1 ml. of molybdenum reagent is added to 2 ml. of phospholipid dispersion containing not more than 2 μmole of lipid in a glass stoppered test tube or a 10 ml. measuring cylinder. The test tube is heated on a boiling water bath for about two minutes. The solution is allowed to cool for about 5 minutes and 3.5 ml of chloroform-methanol (9:1) is added to it. The tube is stoppered and the two phases mixed by slow rocking motion manually or on a cyclo-mixer. The tube is allowed to stand for about 20 mins. when the two clear phases separate out. The lower blue phase is removed by aspiration or with a separating



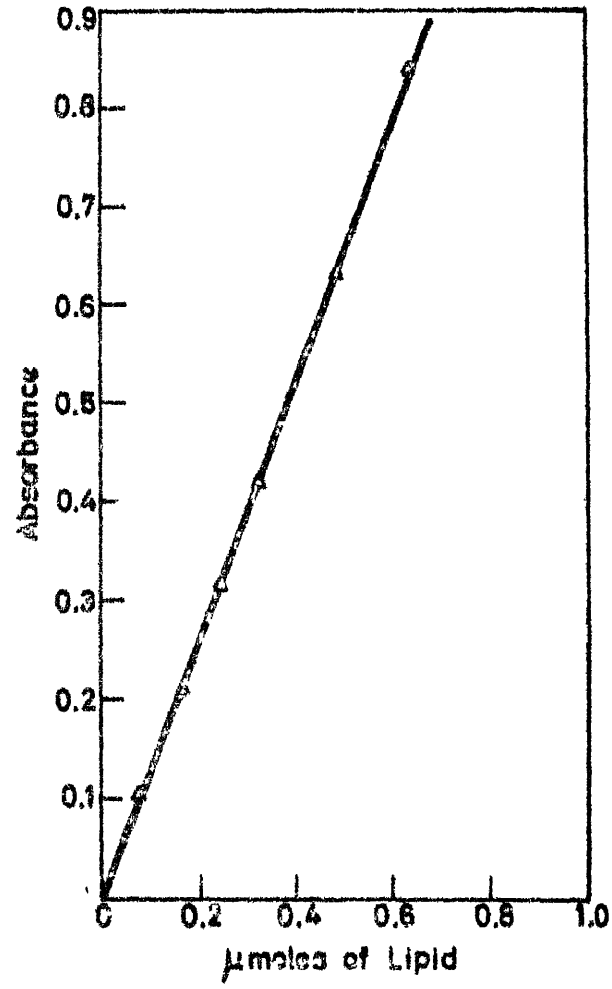


Fig. 1. Absorbance of chloroform phase as a function of (a) volume of reagent in ml used per ml of aqueous dispersion; (b) time of incubation (in min) at 100°C of the aqueous dispersion with the reagent.

Fig. 2. Absorbance of chloroform phase as a function of the quantity of *sn*-3-phosphatidylcholine (in µmol; volume of $CHCl_3$ phase = 3.5 ml).

funnel. The absorbance of this solution is read against a blank of chloroform at 710 nm. in a 10 mm cuvette.

(v) The absorbance is linear with the concentration of lipid in absorbance range 0.05 - 1.0 with a standard deviation of 3%. A typical plot of absorbance as a function of lipid concentration is presented in Fig. 2. The absorbance does not change for at least 30 mins after separation of phases.

(vi) The effect of some solutes which may be commonly present in phospholipid dispersions on phosopholipid estimation by this method is presented in Table 1. It is evident that there are no significant deviations.

(vii) No differences in estimation of an oxidized and fresh lipid were detected. This is understandable since the experimental conditions during estimation are expected to cause lipid oxidation.

(viii) The major source of error is a failure to bring the complex to an equilibrium distribution between the two phases. It is important, therefore, that the two phases be mixed as thoroughly as possible. Vigorous mixing however leads to formation of emulsions which do not clear on standing. In some cases separation can be achieved easily by centrifugation for 5-10 mins.

(ix) With this reagent the method is sensitive down to a lipid concentration of 0.1 μmole/ml. Still lower concentrations may be estimated by using smaller volume of chloroform and microcuvettes for absorbance measurement.

Table 1

Effect of Various Additional Solutes, in the Dispersion, on the Estimation of sn-3-Phosphatidylcholine

Solvent	Absorbance*
Distilled water	0.490
5% ethanol + water	0.490
0.05 M $CaCl_2$	0.487
0.10 M NaCl	0.490
0.5% sodium deoxycholate solution	0.485
0.01 M sodium phosphate	0.490
0.5% bovine serum albumin + 0.10 M NaCl	0.490
0.5% cholesterol + 0.10 M NaCl	0.492
0.5% Ceramide + 0.10 M NaCl	0.491

*Average of three measurements.

Table 2

Extinction Coefficients of Various Phospholipid Molybdnum Blue Complexes at 30°C, 760 nm, solvent : dichloromethane-methanol (8:2). Molar lipid concentration determined by method of Fiske & Subbarow (3)

Lipid	Extinction coefficient* $\mu mole^{-1}\ cm^{-1}$
Egg phosphatidylchotine	1.35 ± 0.005
Lysophosphatidylcholine	0.84 ± 0.02
Cardiolipin	0.92 ± 0.02
Sphingomyelin	1.1 ± 0.03

*Absorbance/quantity of lipid (μmol), path length = 1 cm.

The work described above was done in the summer months when the ambient temperature is about 35-45°C. However in the course of latter work when one prepared a fresh reagent it was already winter and the ambient temperature fell to 16-20°C. One could even visually observe that the reagent had a fainter color. The absorbance of the complex formed with a known quantity of phospholipid was also less. A systematic study was therefore carried out to find the effect of various experimental parameters during reagent preparation on the phospholipid estimation. It was also felt that the problem of emulsion formation with chloroform needs more attention. Although the use of chloroform methanol mixture helps considerably to reduce the formation of emulsions, it does not eliminate it totally. The results of our systematic studies along these lines may be summarized as follows :

(1) The reagent has an absorbance maxima at 710 nm. The A_{710nm} of the reagent increases with the time, temperature, volume of mercury and the concentration of ammonium molybdate solution in HCl used for the reduction.

(2) The amount of complex formed does not depend on the concentration of ammonium molybdate in sulfuric acid solution, However, it increases with the concentration of sulfuric acid in the reagent and is maximum for sulfuric acid concentrations above 30%. If the concentration of sulfuric acid in the reagent is above 65%, emulsions may

form under the conditions used which clear with difficulty.

(3) When reagents containing 50% sulfuric acid are used the amount of complex formed increases with A_{710nm} of the reagent and is maximum for A_{710nm} = 0.45 and above. Therefore, for reproducible results with different reagents one must ascertain that the absorbance of the reagents is atleast 0.45.

(4) Organic solvents like dichloromethane, tetrachloromethane, benzene, petroleum ether and cyclohexane can be used to extract the complex and form emulsions less often. Benzene, petroleum ether and cyclohexane are more convenient to manipulate since the upper organic layer can be separated easily without the need of a separating funnel by careful decantation. The absorbance of the complex does not vary appreciably in these solvents.

On the basis of these observations we arrived at the following modified procedure for molybdenum reagent preparation: 4 gm of ammonium molybdate (AR) is dissolved in 50 ml of hydrochloric acid (35%) and reduced by magnetic stirring with 2 ml of mercury in a 100 ml conical flask for about 45 min. The dark green solution is filtered and added to 100 ml of sulfuric acid (75%) and allowed to cool. The reagent thus prepared has an absorbance of greater than 0.50 at 710 nm using water as reference, when the temperature of reduction is in the range 20-45°C.

The values of the extinction coefficient depend on the solvent used, the fatty acid composition of the phospholipid and the temperature at which the estimation is carried out. Therefore, when one estimates a particular phospholipid, one must determine the extinction coefficient under defined conditions. Thus when, one uses the reagent to determine concentration of a synthetic phospholipid, one gets an exact number. If one has a mixture of phospholipids one gets an estimate, because all one can use is a mean extinction coefficient.

The blue color in the organic layer is due to the formation of a phospholipid-molybdenum blue complex (7). However, I have observed that if a molybdenum blue solution is prepared by reduction of ammonium molybdate with mercury in sulfuric acid solution, very little of the complex is formed, even with fairly concentrated solutions. This points out that hydrochloric acid has a definite role in the complex formation. It is known (8) that when ammonium molybdate is reduced in HCl solution ammonium pentaoxymolybdate $(NH_4)_2$ $(MoOCl_5)$ is formed. The green solution of the reagent containing 8-9 M sulfuric acid probably contains an equilibrium mixture of $(MoOCl_5)^{2-}$ and $(Cl_5OMo\begin{smallmatrix}\diagup\ O\ \diagdown\\ \diagdown\ O\ \diagup\end{smallmatrix}MoOCl_5)^{4-}$. The blue lipid complex is due to the change in oxidation state of Mo^{V} to a mixture of oxidation states +5 and +6 on complex formation.

Molybdenum blue is known to be a complex mixture of these two oxidation states. Fatty acids do not form the blue extractable complex, presumably because they lack phosphate group which is the liganding group in this complex. The fatty acid chains are responsible for the solubility in the organic solvent. Glycerophosphoryl compounds, other polar phosphate esters and phosphate ions do not interfere in this estimation because if any complex is formed it is not soluble in the organic phase.

The molybdenum reagent described here can be used to estimate phospholipids in other solvents also. I have found it extremely useful in monitoring the elution of phospholipids from chromatographic columns. About 2 ml. of the solution is mixed with 2 ml of the reagent and 2 ml. of water, the intensity of the blue color in the organic phase indicates the relative concentration of the lipid in the various fractions. It can also be used as a spray reagent for TLC plates instead of Dettmer and Lester's reagent.

A few other methods based on similar principle have come to my notice since the completion of this work. In one of the methods published almost simultaneously with this method, the ferrothiocynate complex of the phospholipid is used for colorimetic determination of phospholipid concentration (9). This method involves prior extraction

of the phospholipid from the aqueous solution into chloroform and subsequent formation of the complex by addition of an aqueous ammonium ferrothiocynate solution. Perhaps, as in our method, if one uses a more concentrated solution of ammonium ferrothiocynate one may be able to estimate phospholipids in aqueous dispersions directly without prior extraction. The author has not investigated the interference by other solutes systematically, but has observed that there should be no trace of detergent in the apparatus used, In the methods used for estimation of free fatty acids in biological samples (10,11) phospholipids interfere (10). Therefore, these methods can be used for the estimation of phospholipids also in the absence of free fatty acids. Perhaps one can estimate phospholipids and fatty acids simultaneously if one uses one of these methods alongwith our method.

References

1. Briggs, A.P. (1924) J. Biol. Chem., 59, 255.

2. Leiboff, S.L. (1931) J. Lab. Clin. Med., 16, 495.

3. (a) Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem., 66, 375.

   (b) Dettmer, J.C. and Well, M.A. (1969) Methods Enzymol., 14, 384.

   (c) Bartlett, G.R. (1959) J. Biol. Chem., 234, 466.

4. Chen, P.S., Toribee, T.Y. and Warner, H. (1956) Anal. Chem., 28, 1756.

5. London, E. and Feigenson, G.W. (1978) Anal. Biochem.88,203.

6. Raheja, R.K., Kaur, C., Singh, A. and Bhatia, I.S. (1973) J. Lip. Res., 14, 695.

7. Galanos, D.S. (1970) Lipids, 5, 573.

8. Kundu, S.K., Chakarvarty, S., Bahadur, N. and Saha, H.K. (1977) J. Lip. Res. 18, 128.

9. Stewart, J.C.M. (1980) Anal. Biochem. 104, 62.

10. Duncombe, W.G. (1963) Biochem. J., 88, 7.

11. Itaya, K. (1977) J. Lip. Res. 18, 663.

Most of the work presented in this chapter has been published as

1. Hallen, R.M. (1980) J. Biochem. Biophys. Methods,2,251.

2. Hallen, R.M. (1981) J. Biochem. Biophys. Methods,4,241.

Chapter III

For the proposed studies, one needed enough phospholipids readily available. Supply of cardiolipin from the only source in India - CSIR Center for Biochemicals - was irregular. Relying entirely on imported chemical has its difficulties. It takes a long time and 'some' effort if one has to prevent the lipid from getting damaged during storage at the Customs. If one's imported stock somehow gets damaged during storage in one's own laboratory, one has to wait for months to get replacement. I ,therefore, decided to learn how to make pure phospholipids in our own laboratory. Synthesis of cardiolipin (1,2) was not accomplished when I started the work. In any case, not being a synthetic organic chemist, I would prefer isolation from natural sources. Table I summarizes the phospholipid composition of different bovine organs. A look at the table clearly indicates that bovine heart is the ideal source of cardiolipin.

The method described below has been used to extract lipids from bovine heart for our investigations. It combines different aspects of the several published methods and incorporates suitable modifications. Some understanding of the basic processes involved in the isolation and purification procedures has evolved during the course of the work. The choice of the method is based on this understanding. To start with, the choice of the methods and the design of the

Table I

Major phospholipids composition of t' e various bovine organs which may be used for lipid extraction

Phospholipid	Tissue				
	Brain	Heart	Liver	Kidney	Spleen
PC	29.2	24.2	54.2	32.4	36.0
PE	12.1	16.5	9.4	28.6	24.5
PI	3.2	4.1	7.9	7.2	4.3
PS	16.6	2.4	4.2	7.5	12.0
SM	12.8	11.5	5.8	13.7	14.5
DPG (CL)	0.7	8.9	4.1	6.5	0.3
PA	0.5	2.2	2.2	0.2	0.6

From 'Form and Function of Phospholipids' Ansell et al. (eds.) Elsevier Scientific Publishing Company, Amsterdem.

modifications were guided by local experimental constraints, but eventually, what emerged is an eclectic method, which is an overall improvement in technique. The method is presented along with the rationale for the choices. The method proceeds in three main stages; (i) Extraction, (ii) Concentration, (iii) Isolation and purification.

Stage zero : Material for extraction :

Fresh bovine hearts obtained from the local slaughter house within a few hours of slaughter are freed from membranes, fats and connective tissue and stored frozen below 0°C till used. The tissue is cut into small pieces and passed through a hand operated meat mincer with 2 mm holes. The minced tissue is washed with chilled water and filtered through a double layer of cheese cloth, till the filtrate is only faintly pinkish. Most of the erythrocyte cells, which contain significant proportion of phosphatidylserine (PS) (19.3%) are thus separated. PS is known to interfere with the final purification of cardiolipin (CL).

Stage One : Extraction from tissue :

(i) Choice of the technique used : The method most often used for extraction of phospholipids from biomaterials is due to Folch et al (3). In this method the tissue is homogenised with 20 or more volumes of chloroform-methanol (2:1) mixture in a blender. The blending is presumably

necessary to disperse the bound water in the tissue which retards the extraction process. The volume of the solvent used is large. Otherwise, the blending has to be done for a longer period which may cause oxidation of the extracted lipids. A method which uses smaller volume of extracting solvent was introduced by Bligh and Dyer (4). In this method the biomaterial is homogenised first with chloroform-methanol ((1:2) by volume) yielding a monophasic solution. To separate lipids from non-lipids, chloroform and water are added to the solution with another short blending, yielding a biphasic system, in which the lower organic phase contains most of the lipids. The higher percentage of methanol helps in breaking the ionic and hydrogen bonds that hold the phospholipids in biomembranes. This makes this methanol rich solvent more efficient in extraction. Other solvent mixtures have also been used (5), but Schmid (6) has shown in an elegant and systemtic study that chloroform-methanol is a better extracting solvent mixture. In adopting this method, I had a funny problem. The bearings of the commercial blenders of local make in our laboratory are attacked by chloroform and get jammed in no time. So, I adopted an alternative method, due to Pangborn (7). The bound water is first extracted with acetone, which dehydrates the tissue and extracts most of the neutral lipids, glycerids, and some loosely bound phospholipids (8). The lipid is then

extracted with chloroform-methanol for a longer period without blending. It was also thought worthwhile to investigate the composition of chloroform-methanol mixture to achieve maximum yield. For this purpose, the minced tissue is extracted at 4°C with acetone for 3 hours, homogenised in a blender and then dried. 50 gm aliquots of this powder were then extracted with 200 ml of different chloroform-methanol mixture for six hours each. The total phospholipid concentration in the extracts determined by the method described in Chapter II, are presented in Table II. Chloroform-methanol (1:1) gives about the maximum yield. Homogenisation with acetone which is free of the problem faced with chloroform (i.e., jamming of blenders) accelerates the extraction in this step.

(ii) The method : In the actual extraction, the washed mince is extracted with 4 volumes of acetone for about 12 hours at 4°C. The minced tissue is then homogenised with minimum volume of acetone in a blender for about a minute and filtered with a double layer of cheese cloth on a Buchner funnel. The extracted tissue is allowed to dry for about an hour under a fan. It is then extracted with 3 volumes of chloroform-methanol (1:1) for 12 hours with intermittant shaking at 4°C. The extraction is repeated once more with the same quantity of solvent, after filtering off the first extract.

TABLE II

Extraction efficiency of various solvent mixtures 50 gms of washed and acetone washed tissue extracted with 200 ml. of the solvent

Solvent Composition	Phospholipid Concentration Mole/ml.
Methanol	2.0
Methanol:Chloroform (9:1)	2.25
Methanol:Chloroform (8:2)	3.0
Methanol:Chloroform (7:3)	3.5
Methanol:Chloroform (1:1)	4.0
Methanol:Chloroform.HCl(1N) (7:3:1)	3.2
Methanol:Chloroform.NH_3(1N) (7:3:1)	2.3

Stage Two : Concentration of the extract :

(i) Choice of technique : In the published methods (Table III), the tissue extract is concentrated either by solvent evaporation or the phospholipids are precipitated and redissolved in chloroform. The concentrate is then processed further to separate the different lipids from one another. This is necessary because the extracting solvent is usually not appropriate for isolation and purification of phospholipids. There was some difficulty in adopting these procedures mostly because of local experimental constraints. I, therefore, tried a new approach. It looks simple, is elegant in principle and it works. The crux of the method is to add enough water to chloroform-methanol (1:1) extract of the phospholipids. A chloroform phase (containing at most 2-3% methanol and water) separates out as the lower phase and methanol goes into the aqueous phase. Thus the concentration of the lipid solution is achieved not by removal of solvent by evaporation but by extracting off a part of the solvent mixture into an immiscible phase (water). This chloroform solution of a mixture of phospholipids is ready for further separation into individual phospholipids by column chromatography. It can be dried by some drying agent or can be applied to the column right away.

This method is, in practice, much more economical and straightforward than the methods used hitherto. It is also

ether mixture after concentrating the chloroform-methanol extract. The precipitate is then separated out by centrifugation at -10°C. I got a poor yield in this method perhaps because the Remi centrifuge in our laboratory went down only to -4°C. The proteolipid is perhaps considerably more soluble at this temperature. Precipitation as Ba or Cd salt is complete only in a fairly polar solvent. Pangborn (7) precipitates cardiolipin as Ba^{2+}-salt in methanol whereas Takahashi et al. (10) use methanol-ether (3:1). These methanol-rich solvents are however not good for extraction of phospholipid from the tissue. On the other hand, precipitation of Ba-salt from the 1:1 chloroform-methanol mixture used by us for efficient extraction, is incomplete. This makes the yield low. These problems are all circumvented by the method used in this work.

(ii) The method :

An equal volume of an aqueous solution (pH 8.5, 0.1 M tris, 0.1 M NaCl, .01 M $CaCl_2$) is added to the chloroform-methanol extract of the phospholipid mixture and the phases are allowed to separate. The lower chloroform phase is washed once with half its volume of the same aqueous solution and is allowed to stand at 4°C till the two phases separate out totally. The lower chloroform phase is separated with a separating funnel and stored below -10°C. An alternative to using a separating funnel is the use of phase

separating filter papers manufactured by Whatman, U.K. This is a more convenient method, but it came to our notice after the completion of this work.

Stage Three : Separation and purification of phospholipids :

(i) Choice of techniques : Two distinct techniques have been described in the literature, (a) selective precipitation and extraction (7), (b) partition or adsorption chromatography (11-14). The second method is, of course, the method of choice. Precipitation and solvent extraction have to be carried out several times to achieve good separation. Each step involves some loss of lipid. In addition, the lipid is exposed to air more often, unless one carried out the whole experiment under N_2. Column chromatography for phospholipids has been described on DEAE cellulose (11), silicic acid (12,13) and Aluminium oxide (14). DEAE cellulose requires considerable skill for packing. The elution scheme is also very elaborate. Separation of ox-heart lipids and their elution behaviour on a silicic acid column has been studied elaborately by Gray and MacFarlene (12). Nielson (15) and Shimojo et al. (16) have studied the behaviour of Ca, Mg and Na salts of cardiolipin on silicic acid columns. The general conclusions from these studies are that calcium-cardiolipin is eluted even with chloroform-methanol mixture containing less than 10% by volume of methanol, whereas, Sodium-cardiolipin is eluted only with 60:40 chloroform-methanol.

Phosphatidylethanolamine (PE) is eluted from silicic acid column with 4:1 chloroform methanol and phosphatidylcholine (PC) is eluted at a ratio 3:1. In order to ascertain the separation of PE and PC, I investigated the adsorption behaviour of these two phospholipids on silicic acid in the following way : solutions of PC (1 millimole/ml) and PE (0.5 millimole/ml), 35 ml each, is added separately to 15 gm of silicic acid. Various amounts of methanol and water are then added to this solution. The percentage of phospholipid desorbed is calculated from the phospholipid concentration in the supernatant. The results are presented in Table IV. It is clear that there is no clear cut separation of PC and PE on silicic acid, if their concentrations are comparable. However, two broad fractions, one containing mainly PE and the other PC can be eluted by chloroform-methanol (75:20) and chloroform-methanol-water (60:40:10). One, therefore, has a simple primary separation of these three major phospholipids.

In the final purification of cardiolipin, one converts Ca-cardiolipin to Na-cardiolipin by treatment with EDTA and Na_2SO_4. When this solution is applied on another silicic acid column, the pigments and the neutral lipids will elute first in chloroform-methanol 80:20 together with traces of PE, which coelutes with Ca-CL, while pure Na-CL is eluted with a mixture of chloroform-methanol-water (60:40:10).

TABLE IV

Adsorbtion behaviour of phosphatidylcholine and phosphatidyl ethanolamine on silicic acid in various chloroform-methanol-water systems

Solvent Composition	% Phospholipid Desorbed	
	PC	PE
Chloroform	0.005	0.007
Chloroform-methanol (9:1)	0.008	0.06
Chloroform-methanol (75:25)	1.7	17
Chloroform-methanol (65:35)	16	30
Chloroform-methanol-water (65:35:2)	56	60
Chloroform-methanol-water (65:35:3)	99	100

The method of Shimojo and Ohno (13) separates Ca-CL from the pigments and neutral lipids, by first eluting the latter with pure chloroform and then eluting Ca-CL with 98: 2 chloroform-methanol mixture. In my experience, I found it to be a rather difficult separation. This is so, perhaps because the polarity of the two solvents are very close. Their method has the further difficulty that removal of the extracting solvent ethanol-ether mixture is somewhat cumbersome because of the higher boiling point of ethanol and the brisk effervescence which invariably ensues.

Phosphatidylcholine can be purified by precipitation from acetone rich solvent easily while PE can be purified on another silicic acid column, where the separation from PC is more efficient because of its lower relative concentration.

(ii) The Method :

(a) Primary Separation of Phospholipids

Washed silicic acid (60-120 mesh, about 30 gm/mmole of phospholipid; activated at 120° for 20 hours) is packed in a wide column fitted with G-1 sintered glass disc as a slurry in chloroform. The washed chloroform phase of the extract, mixed with 1/20 of its volume of methanol, is passed through it. When all the extract has been passed the column is washed down with two col. vols. of chloroform methanol (9:1). The two pooled eluates contain Ca-CL, Ca-PI, Ca-PA, the neutral

lipids and the pigments (Fraction I). Most of the phosphatidylethanolamine is then eluted with chloroform methanol (65:30) together with traces of acidic phospholipids and neutral lipids and phosphatidylcholine, in about five column volumes (Fraction II). Finally phosphatidylcholine is eluted with chloroform-methanol-water (65:35:3) mixture in about four column volumes with traces of phosphatidylethanolamine and some lysophospholipids (Fraction III).

Final Purification

(i) Cardiolipin : To the cardiolipin fraction (Fraction I) is added half its volume of methanol and half its volume of an aqueous solution of disodium EDTA (0.02M), sodium chloride (0.1M) at pH 5.5. The resulting emulsion is mixed well and allowed to settle till the two phases separate out. The washing is repeated once with equal volume of EDTA solution and once with distilled water. To the resulting turbid chloroform phase is added one tenth its volume of methanol and it is passed through a silicic acid column (100-200 mesh, 125 gm/m mole lipid). The column is eluted with two column volumes of chloroform-methanol (85:15) to wash out all traces of phosphatidylethanolamine, neutral lipids and other acidic phospholipids. Cardiolipin can then be eluted as its sodium salt with chloroform-methanol-water (60:40:2) in about three column volumes. The eluate is then evaporated under vacuum to 1/20th its volume and tested on a

TLC plate. If any impurities are present they are removed either by precipitation from methanolic solution as Barium salt or on another silicic acid column.

(ii) Phosphatidylethanolamine : The phosphatidylethanolamine fraction (Fraction II) is pooled with the 85:15 eluate from the cardiolipin purification column, and reduced to one tenth of its volume. It is then mixed with equal volume of chloroform and applied on a silicic acid column (100-200 mesh mesh). Phosphetidylethenolamine is adsorbed and the residual acidic phospholipids and carotenoids are eluted out. The column is washed once with one column volume of chloroform methanol (90:10) and then phosphatidylethanolamine is eluted with 4 column volumes of chloroform methanol (70:35).

(iii) Phosphatidylcholine : The phosphatidylcholine fraction (Fraction III) is reduced to one twentieth its volume under vacuo at less than 40°C temperature. To the concentrate is added ten times its volume of acetone, with constant stirring. The mixture is allowed to cool down to below 0°C in a freezer for about 3-4 hours. The precipitated phospholipid is dissolved after filteration or centrifugation, in minimum volume of petroleum ether and reprecipitated with ten volumes of acetone below 0°C to obtain pure phosphatidylcholine free from lysolipids and phosphatidylethanolamine.

Monitoring of the Elution from Columns

The total phospholipid content of the eluate at any particular stage of eluation can be very rapidly and conveniently monitored by the molybdenum reagent described in Chapter II.

In the primary separation and the purification columns the eluates must be tested with ninhydrin to ensure good separation of phosphatidylethanolamine from the other lipids.

Vacuum Evaporation Technique

Since liquid nitrogen was not readily available one had to improvise some method for vacuum evaporation. We have a cryostat in our laboratory which can cool down to -23°C. This cryostat was filled with a ethylene glycol-water (1:1) mixture. A 2 liter glass bottle fitted with a standard glass joint was immersed in it with the trap vapour fitting as shown in figure 1. The vapors from the Buchler flash evaporator were led through the metal coils immersed in the bath to this trap. The vapors are thus more efficiently condensed and the solvent collects in the bottle. With this system about 600 ml of solvent can be removed each time in about 90 mins. using a rotary vacuum pump at 1 mm Hg. pressure.

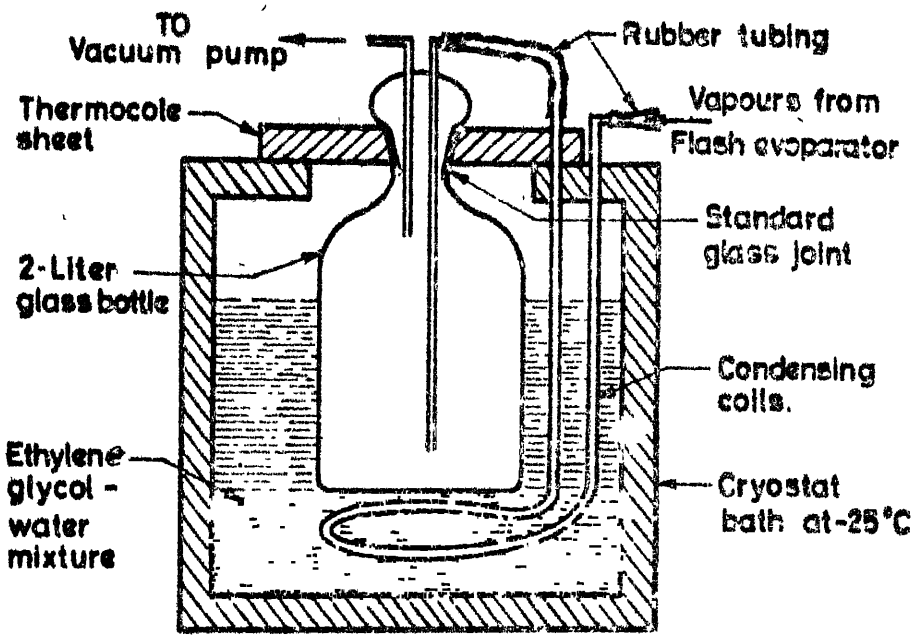


FIG. 1. SCHEMATIC REPRESENTATION OF THE LOW-TEMPERATURE TRAP USED FOR VACUUM DISTILLATION OF COLUMN ELUATES.

Yield and Purity

From about one Kg. of washed minced tissue the yields of the pure phospholipids are : Cardiolipin 1 m mole, Phosphatidylethanolamine 6 m mole and phosphatidylcholine 10 m mole. The phospholipids give a single spot on silica gel TLC developed with chloroform methanol water 65:25:4 and chloroform methanol ammonia 70:30:4, which is identical with that of cardiolipin obtained from Sigma and CSIR Center for Biochemicals, Delhi.

Most of the materials, silica gel and solvents, can be recycled. The aqueous upper phase can be distilled to get methanol for washing silica gel, and the distillate from the chloroform methanol (9:1) mixture can be used for higher polarity elution, while the distillates of higher polarity eluates can be used for extraction of tissue.

References

1. Kates, M. 1977. Synthesis of stereoisomeric phospholipids for use in membrane studies. In Methods in Membrane Biology, Vol. 8. Korn, E.D. editor, Plenum Press. New York. London : 219-290.

2. Raimrez. F, Ioannon, P.V., Marěcek, J.F., Dodd, G.H. and B.T. Golding (1977). Synthesis of phospholipids by means of cyclic enediol pyrophosphates. Optically active, monovalent and Divalent salts of diphosphatidylglycerol (Cardiolipin). Tetrahedron. 33 : 599-608.

3. Folch, J., Lees, M. and H. Sloan Stanly. 1957. A simple method for isolation and purification of total lipids from animal tissues., J. Biol. Chem. 226: 497-503.

4. Bligh, E.G. and N.J. Dyer. 1959. A rapid method for total lipid extraction and purification, Can. J. Biochem. Physiol. 37:911-914.

5. Zahler, P. and V. Niggli. 1977. The use of organic solvents in membrane research. In Methods in Membrane Biology. Vol. 8. Korn, E.D. editor, Plenum Press. New York. London: 1-50.

6. Schmid, P. 1973. Extraction and purification of lipids II. Why is chloroform-methanol such a good lipid solvent Physiol. Chem. Phys. 5. 141-145 and 5, 151-157.

7. Pangborn, M.C. 1945. A simplified preparation of cardiolipin with a note on purification of lecithin for serological use. J. Biol. Chem. 161:71-82.

8. Lucas, C.C., Pettison, I.M. and J.H. Ridout. 1958. Solubility of tissue phospholipids in acetone. Arch. Biochem. Biophys. 78:546-554.

9. Eichberg, J. and J.D. Burnham. 1970. Novel preparation of cardiolipin from beef heart. J. Lip. Res. 11:386-388.

10. Takahashi, T., Hahrmoto, T. and I. Hare. 1967. A rapid method for preparation of cardiolipin by ion exchange, Bull. Soc. Chim. Biol. 49:65-68.

11. Rouser, G., Kritchvesky, G., Heller, D., and E. Lieber. 1963. Lipid composition of Beef brain, beef liver and the sea anemone. Two approaches to quantitative fractionation of complex lipid mixtures. J. Am. Oil. Chem. Soc. 40:425:454.

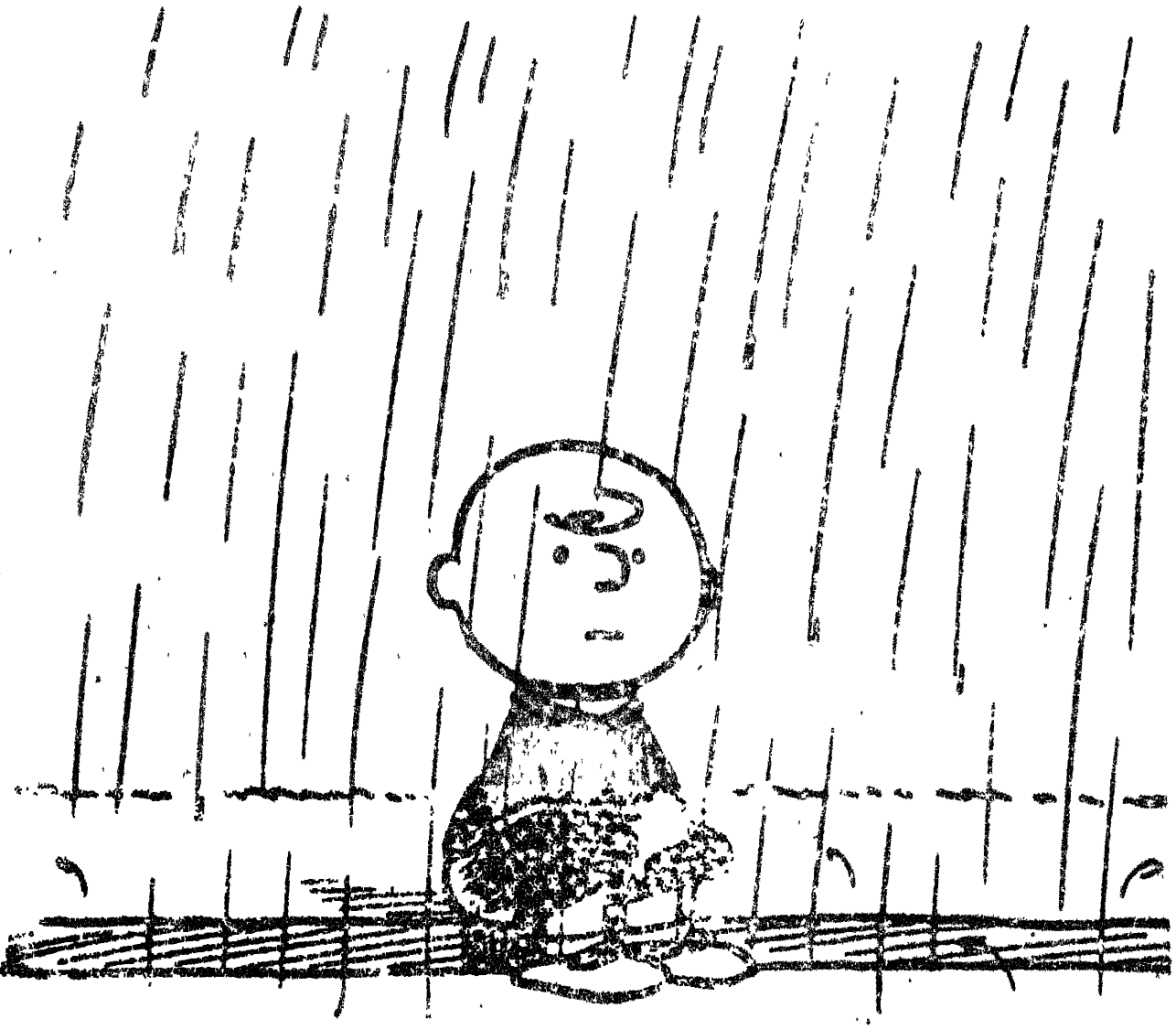
12. Gray, G.M. and M.G. Macfarlane. 1958. Separation and composition of phospholipids of ox heart. Biochem. J. 70:409-435.

13. Shimojo, T. and K. Ohno. 1966. Semiquantitative purification of cardiolipin by column chromatography. J. Biochem. (Tokyo). 60:467-469.

14. Marinetti, G.V. 1967. Lipid chromatographic analysis. Vol. 1, Marcel. Dekker, Inc., New York.

15. Nielson, H. 1974. Silica gel column chromatography of acidic phospholipids, Effects of metal ions of adsorbent and phospholipids on elution profiles. J. Chromatgr. 89:259-273.

16. Shimojo, T., Kanoh, H. and K. Ohno. 1966. Elution behavior of acidic phospholipids on column chromatography. J. Biochem. (Tokyo) 60:251-255.



Chapter IV

As discused in the introductory chapter the size of phospholipid vesicles can be an important parameter in their physico-chemical studies. One needs, therefore, a method by which one can prepare phospholipid vesicles of different sizes or size distributions conveniently and reproducibly. The size or size distribution of vesicles depend on the method of their preparation. Presumably a particular size distribution achieved is kinetically stable.

In the last few years many different methods for preparation of phospholipid vesicles have been developed. The impetus originates from the application of unilamellar vesicles as drug carriers. Their internal volume to lipid ratio is large therefore they are more efficient in encapsulating drugs. These methods can be classified broadly into two sets as follows :

Set A	Set B
Methods using multilamellar liposomes as raw material	Methods using small lipid micelles as raw material
1. Sonication (1)	1. Dialysis or gel filtration of a lipid detergent mixture (5-8)
2. Passing through polycarbonate membrane filters (2)	2. Injection of an ethanolic or ether solution of lipid (9-11)
3. Passing through a French Press cell. (3,4)	3. Reverse phase evaporation (12)

There has been no study of the experimental parameters that affect size of vesicles formed by methods of Set A, while for methods in Set B, it has been shown that vesicle size can be controlled by variation of (a) lipid: cholesterol ratio (6) (b) lipid:detergent ratio (8) (c) lipid:ethanol ratio (10). At the time when this work started the only method reported in literature for varying the vesicle size was the variation of lipid concentration in an ethanolic solution which is injected slowly into a large volume of stirred aqueous medium with a microliter syringe. This method is inconvenient for preparation of larger volumes of vesicle solutions because the injection rate is very slow (1-50 µl/min)and the injection is done manually.

Since Bazri and Korn (9) have shown earlier that small phospholipid vesicles comparable in dimensions to the sonicated vesicles are formed when the ethanolic solution is injected rapidly into the aqueous medium, injection rate is also a parameter which controls vesicles size. Hence, the injection rate needs to be carefully controlled to obtain reproducible preparations. One has to, therefore, in this method inject very slowly and carefully the ethanolic solution for about 30 minutes to obtain 20 ml. of vesicle solution.

However, if the injection is mechanically controlled, the method can be both convenient and reproducible. In

addition if instead of one capilliary of the syringe one has an array of capillaries for injection, the injection time can be reduced. A porous disc is an object which can provide an array of capilliaries. These initial ideas led me to the design of the injection device described below. With this injection apparatus the effect of a variety of experimental parameters on the size of the vesicles were investigated. These eventually led me to a plausible mechanism for the formation of vesicles by this method.

Injection Apparatus : The apparatus used for passing the alcoholic solution into the aqueous medium is illustrated in Fig. 1. The device A is machined from a teflon rod. The sintered disc used is grade 4 (porosity 2-5 microns). The disc is adequately tightened so that no solution leaks from the sides. The curved profile below the disc is to ensure easy removal of the air bubbles trapped during the insertion of the device into the aqueous solution. The threading of the rod R facilitates insertion of the device to a desired fixed level for different volumes of aqueous medium. The pressure at the surface of alcoholic solution is maintained constant with the help of the pressure controlling device B and the temperature is held constant by circulating a thermostatically controlled liquid in the glass jacket. The pressure source can be selected according to need and

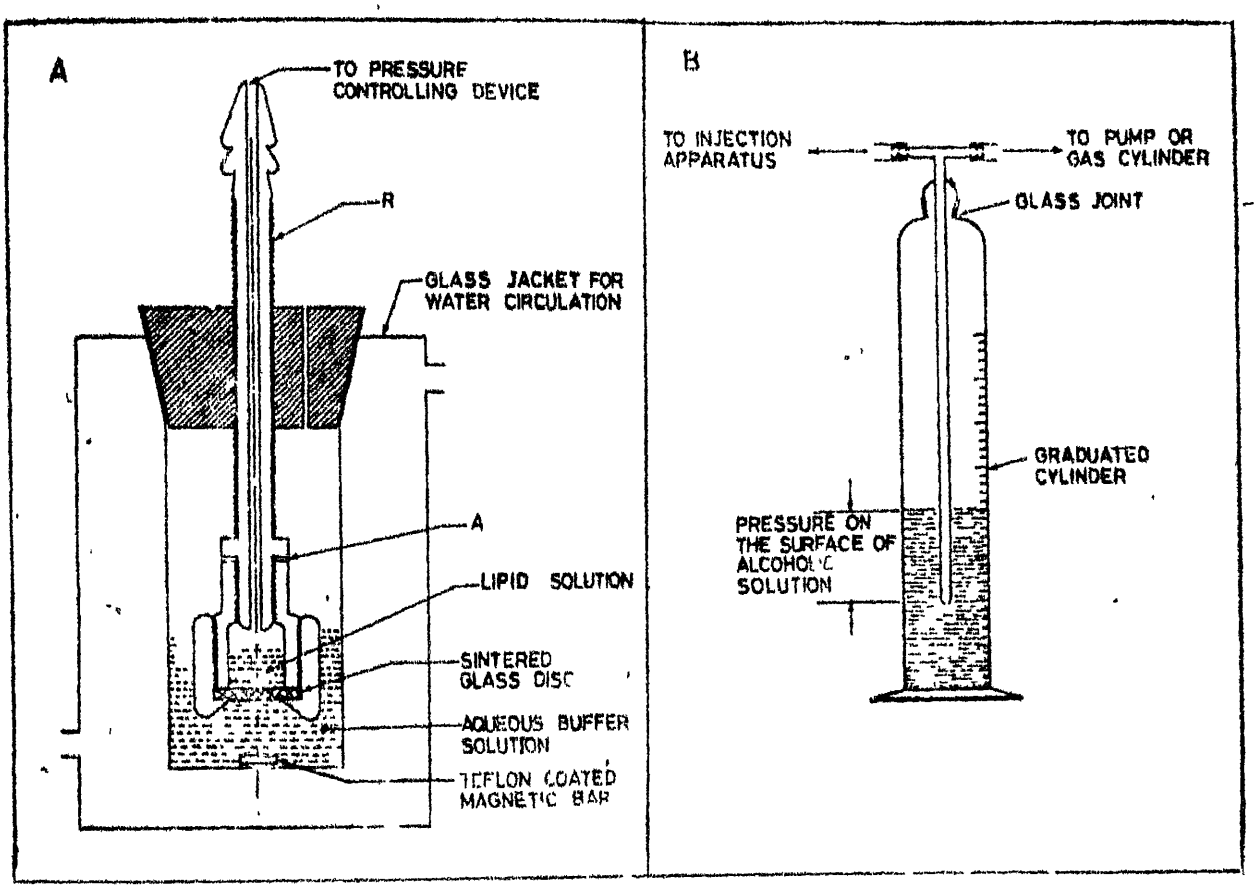


Fig. 1 Schematic diagrams of (a) the injection device (b) the pressure controlling device. Diameter of the sintered disc in the injection device is 2 cm; length of the teflon piece 13 cm.

convenience. A nitrogen gas cylinder, a small vacuum pressure pump or an aquarium pump can be used.

Materials : Chromatographically pure phoshatidylcholine was isolated from hens eggs (13).Phosphatidylethanolamine and Diphosphatidyl glycerol (cardiolipin) were isolated from bovine heart by a method described in Chapter III. All other chemicals used were of analytical reagent grade. Aqueous solutions were prepared in distilled deionized water and filtered through a 0.2 μm cellullose acetate Millipore filter.

Injection : The phospholipid solution is pipeted out into the injection device and the device is lowered into the aqueous solution in the jacketed cell at the required temperature upto a fixed mark on the device. The jacket is tilted slightly to remove the air bubble trapped and the top of the device is connected to the pressure source through the pressure controlling device. If a pump is used, the magnetic stirrer and the pump are switched on simultaneously. The length of the bar used is about the diameter of the disc and stirring rate is controlled so that the bar does not wobble during injection and stirring is uniform. The device is precalibrated for the time required to pass known volumes of various solvents. The injection is stopped when about 90% of the solution has passed through by switching of the pressure source. After every injection the device is dipped

into the solvent passed and the disc is not allowed to get dry between injections. This prevents formation of any bubbles in the solution during injection which affects the size distribution of the vesicles formed. The typical injection rate of alcoholic solution is about 7-20 minutes/ml depending on pressure and temperature.

Size estimation : The size distribution of the vesicles formed was estimated from optical dissymmetry measurements and negatively stained electron micrographs.

Optical dissymmetry measurements were carried out on a Brice Phoenix 2000 light scattering photometer at 546 nm and the temperature of the injection. All solutions were filtered directly into the cylinderical cell through a 0.22 μm Millipore cellullose acetate filter using a Swinnex filtration assembly. The first 5 ml. of the filtrate is discarded because, if this fraction is included, the dissymmetry of the solution increases considerably. It is possible that this is due to some substances being leached out from the filter (14). The phospholipid concentration of all solutions for dissymmetry measurements was below 1 μ mole/ml. Therefore measurements were carried out at one concentration only.

Negatively stained electron micrographs were taken on a Phillips EM 301 electron microscope operating at 60 KV. A drop of the vesicle solution mixed with ammonium molybdate (1%,pH 7.5) was placed on a carbon coated grid and then sucked off using the edge of a filter paper. The grid is

allowed to dry for a few minutes and then examined under the microscope.

Phospholipid estimation : The determination of phospholipid concentrations was carried out using the molybdenum blue reagent. The method is described in Chapter II.

Removal of alcohol : The method used for alcohol removal is a slight variation of the method described by Fry et al. (15). A 2.5x30 cm column of Sephadex LH20 is packed in the buffer used for injection. This column was found satisfactory to remove completely alcohol from 20 ml. of vesicle solution. The column is allowed to run till no more buffer is eluted. 20 ml. of the vesicle solution is then applied slowly and the column allowed to elute similarly till all the solution sinks into the gel. Slight nitrogen pressure is then applied to remove some more solvent. Vesicles can then be eluted with 20 ml of fresh buffer.

RESULTS : The effect of various experimental parameters in this method on the optical dissymmetry of the vesicle solutions is shown in Fig. (2).

About 5-20% of the phospholipid injected is lost during millipore filteration. The percentage of this loss depends on the concentration of lipid solution injected and is maximum for maximum concentration.

The effect of phospholipid composition on the dissymetry of the solution is presented in Table I. Phosphatidylcholine

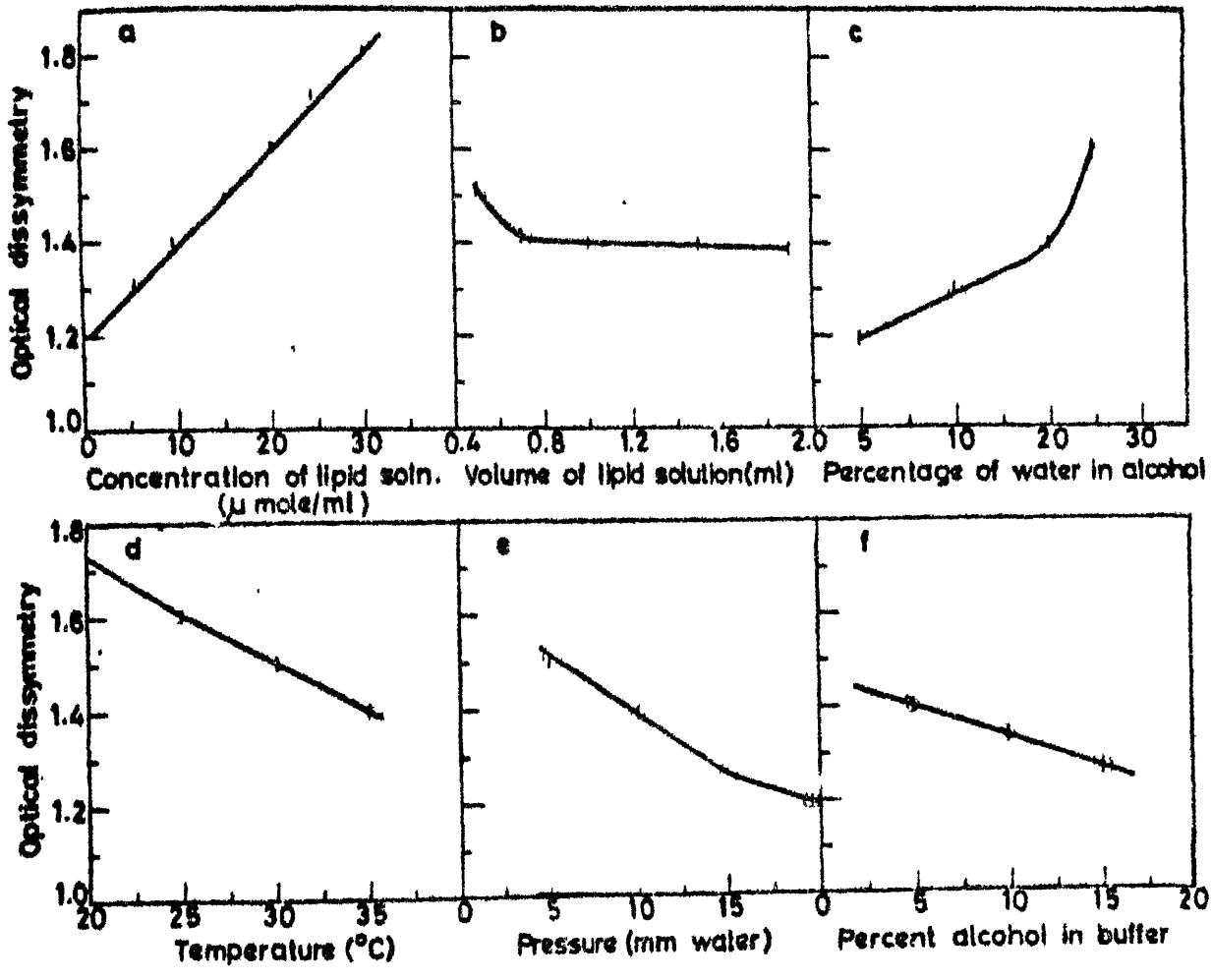


Fig. 2 Plots of dissymmetry of the vesicle solutions vs. (a) the concentration of phosphatidylcholine ethanolic solution injected at 35°C and 10 mm pressure, volume injected 1 ml. (b) The volume of lecithin solution of concentration 9 μmole/ml. injected at 35°C and 10 mm pressure. (c) the percentage of water in the ethanolic solution of lecithin, concentration 9 μmole/ml. injected at 35°C; pressure 10 mm. (d) the temperature of injection of lecithin ethanolic solution, concentration 9 μmole/ml., pressure 10 mm. (e) the pressure of injection of lecithin alcoholic solution concentration 9 μmole/ml. at 35°C. (f) the percentage of alcohol in the aqueous medium in which lecithin alcoholic solution concentration 9 μmole/ml. is injected at 30°C, pressure 10 mm.

The aqueous medium in all these experiments is 20 ml. of 0.1 NaCl, 0.01M Tris. pH 7:5. Dissymmetry values show a spread for three preparations.

Table I

Optical dissymmetry of vesicle solutions prepared by injecting 1 ml of different phospholipids solution at 35°C and 15 mm water pressure to 20 ml of buffer

Phospholipid	Concentration (μ mole/ml)	Optical dissymmetry
sn-3-Phosphatidylcholine (lecithin)	9	1.21 ± .03
Diphosphatidyl glycerol (cardiolipin)	5	1.4 ± .02
sn-3-Phosphatidylcholine-diphosphatidyl glycerol	6-2	1.6 ± .02
sn-3-Phosphalidylcholine-phosphatidyl-ethanolamine	6-2	1.67 ± .02

solutions in various water miscible alcohols and in 1,4 dioxan were injected under same conditions of temperature and pressure The optical dissymmetry factors for these investigations are presented in Table II.

The optical dissymmetry factor increased by about .05 on gel filtration of phosphatidylcholine vesicle solution prepared by this method. About 10% of the lipid is lost during gel filtration. No detectable amount of alcohol is left after gel filtration.

The optical dissymmetry factor for dilute phosphatidylcholine vesicle solutions (ca. 0.5 μmole/ml.) does not change for at least 24 hours although there is a change in the intensity of light scattered at 45° and 135°. Cooling the solution prepared at 35°C to 10°C for about 4 hours and then warming up to 40°C also does not change the dissymmetry factor appreciably. However dissymmetry of diphosphatidyl glycerol vesicle soln. increases on standing for long and also on cooling.

A few typical electron micrographs of a phospholpid solutions of optical dissymmetry 1.67 are shown in Fig. 3. The suspension appears to be fairly homogeneous vesicles of outer diameter (900 $\pm$ 90°A) which is also expected from the dissymmetry measurements.

Discussion : Optical dissymmetry parameter of a solution is a sensitive measure of the size of particles of known shape if the particle dimensions are between .1 λ -.6 λ (λ is the wavelength

Table II

Optical dissymmetry of vesicle solutions prepared by injecting 1 ml of phosphatidylcholine solution in different solvents at 25°C and 20 mm water pressure to 20 ml of buffer

Solvent	Dielectric constant at 25°C	Optical dissymmetry
Methanol	32	1.57 ± .02
Ethanol	24.3	1.5 ± .02
2-Propanol	18.3	1.19 ± .02
1,4-Dioxan	2.209	2.0 ± .02

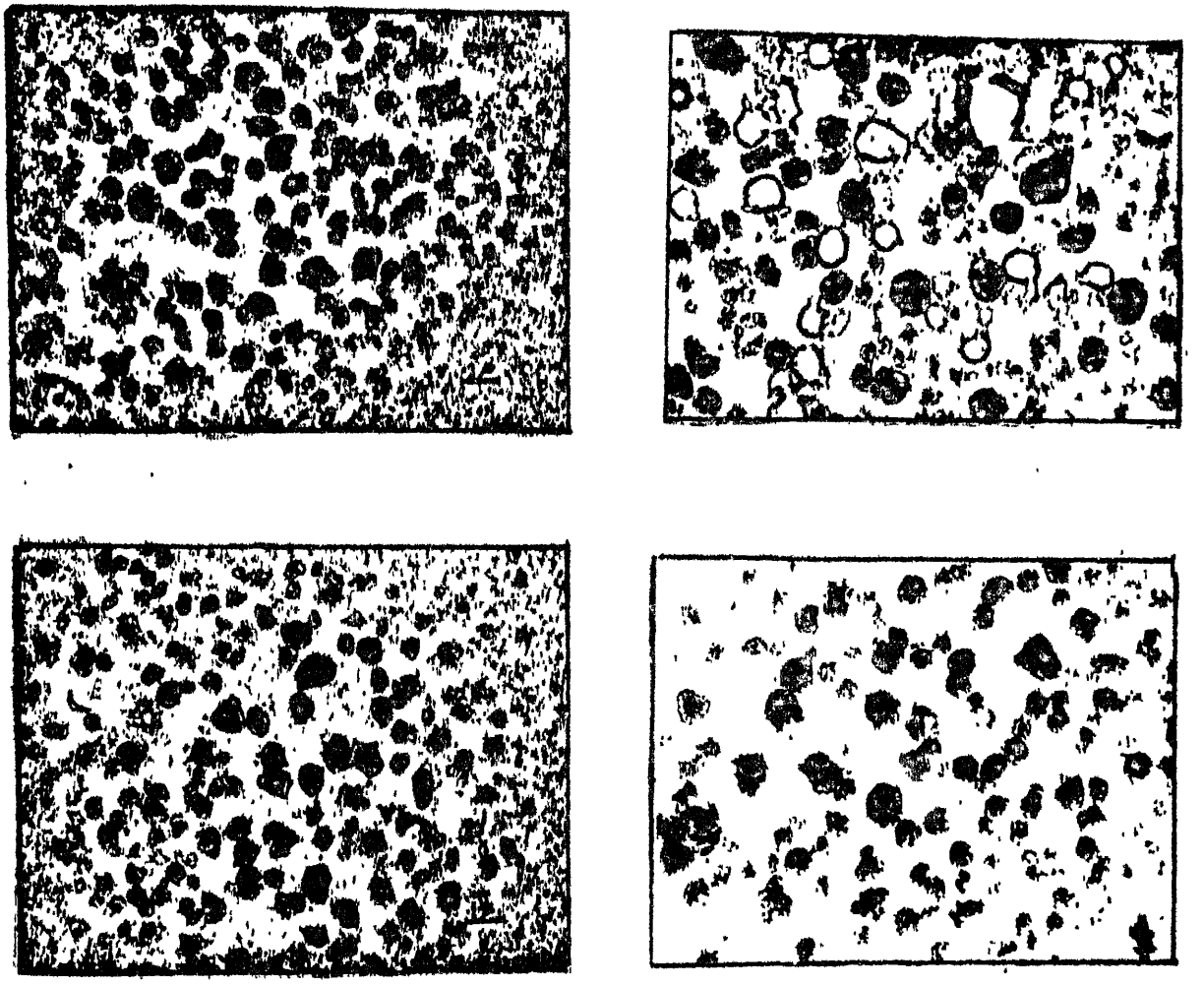


Fig. 3 Some negatively stained electron micrographs of lecithin vesicles of dissymmetry 1.67. Magnification 51000 x

of the scattered light) (16). Seufert (17) has calculated the dissymmetry parameters for spherical shells of different diameters under certain assumptions and used this data to find asolectin vesicle size. The phospholipid systems used in the present study are known to form spherical vesicles, in the aqueous medium used. This is also confirmed from the negatively stained electron micrographs. Therefore calculated optical dissymmetry parameters can be used to estimate the vesicle size if one assumes a single uniform size of the vesicles. However, an exact correlation can not be drawn easily since there is generally a size distribution in vesicle solutions. In addition vesicles may coagulate and fuse with time after formation (18,19). Table IB gives the dissymmetry parameter as a function of size (10).

Phosphatidylcholine molecules aggregate to form micelles in alcohols and alcohol rich alcohol water systems. The size of the micelles increases with the water content (20-22). These observation can be extrapolated to other phospholipids in alcohol solutions. In our experimental setup, the porous disc is covered on one side with alcoholic solution of phospholipids and on the other side with the aqueous medium. An alcohol-water composition gradient is therefore present across the thickness of the disc. The nature of this gradient depends on the pore size, exact composition of the two solvents and the resultant pressure on the alcoholic

Table III

A correlation of the observed dissymmetry values at infinite dilution with the calculated radius of the vesicles (10)

Dissymmetry Z	Radius R_z (nm)
1.02	14.0
1.07	20.0
1.16	26.5
1.20	29.5
1.22	31.0
1.28	34.5
1.38	39.0
1.50	43.3
1.54	44.2
1.62	47.0
1.71	49.0
2.11	56.5
2.19	58.0
2.35	60.0

solution. When greater pressure is applied on the alcoholic solution the gradient will shift more towards the composition of the alcoholic solution. The phospholipid micelles passing through this gradient will grow in size till they come out of the disc into the aqueous medium, where they fuse and rearrange to form vesicles. The above mechanism can explain our results as follows :

(a) Effect of lipid concentration : The concentration of phospholipid can affect the micellar aggregration number and also increase the probability of fusion of greater number of micelles. There is no available information on the effect of concentration on micellar aggregration in these systems. However, one will normally expect an increase. The optical dissymmetry of vesicle solutions prepared under similar other condition is therefore expected to increase with phospholipid concentration. Experimental observat ons agree with this (Fig. 2a).

(b) Effect of volume of lipid solution injected : As one injects more lipid solution into the aqueous medium the concentration of alcohol in the aqueous medium increases, which may affect the size of the vesicles formed. For volumes ranging from 0.7 ml. to 2.0 ml. injected in 25 ml of aqueous buffer there is only a slight decrease in optical dissymmetry but for lower volumes a larger optical dissymmetry is observed. This may be due to an experimental artifact as follows. There

is a time lag between the insertion of the device into the aqueous medium and the switching on of the pressure source. During this time there is a resultant pressure in the opposite direction which builds up a solvent composition gradient across the width of the disc different from the steady state gradient maintained during the latter injection. Vesicles of larger dimensions are therefore expected to be formed during the injection of the first few microliters of the alcoholic solution (Fig. 2b).

(c) Effect of percentage of water in alcoholic solution : Micelles of larger aggregration number are formed in alcohol water mixtures as the percentage of water is increased. Therefore it may be expected that larger vesicles will be formed on injection of phospholipid alcohol solutions containing larger percentage of water (Fig. 2c).

(d) Effect of temperature : Temperature can affect the aggregration number of the micelles and also their fusion probability. Higher temperature may be expected to lower the aggregration number and lower the fusion probability, since at higher average velocity the number of effective collisions will be lower (Fig. 2d).

(e) Effect of pressure on alcoholic solution : An increase in pressure will shift the solvent composition gradient towards greater percentage of alcohol. The aggregration number of micelles entering the aqueous medium will be smaller and

therefore smaller vesicles may be expected to be formed. (Fig. 2e). These observations are also consistent with the method of Bazri and Korn (5) where small phospholipid vesicles are formed by a rapid injection of ethanolic solution.

(f) Effect of percentage of alcohol in aqueous medium : At higher concentration of alcohol in the aqueous medium the fusion probability of the micelles is expected to be lower. Therefore smaller vesicles are formed with increasing alcohol percentage in the aqueous medium (Fig. 2f).

(g) Nature of alcohol and phospholipid : The aggregation behaviour of the phospholipids in alcoholic solutions will depend on the alcohol, the head group of the phospholipid and the fatty acid composition besides the other factors discussed above. Our results indicate on the basis of our hypothesis that micelles with greater aggregration number are formed in alcohols of higher dielectric constant. However dielectric constant alone is not a direct indicator of aggregration behaviour in other solvents e.g. dioxane having very low dielectric constant leads to the formation of larger vesicles. (Table I). Acidic phospholipids form larger vesicles then phosphatidylcholine under similar injection conditions. (Table II).

The method is fairly simple and can be easily adapted to the different individual needs. An alternative possible design for the injection device is to cut a filtration funnel

fitted with a sintered glass disc just above the surface of the disc. Polyethylene porous sheets (for example those marketed by Bel Art products U.S.A.) may also be used. Since the vesicles are formed in fairly homogeneous conditions they are expected to be fairly narrow in size distribution. The conditions of the injection can be appropriately chosen to almost any vesicle size distribution from different phospholipids. A major limitation of the method is that only dilute vesicle solutions, fairly narrow in size distribution, can be prepared. The size of vesicles formed change during injection if more alcohol is injected alongwith the phospholipid for making more concentrated solutions.

The methods for preparation of vesicles of different sizes aim at two different kind of studies:firstly to be used as lysoosmometric carriers of drugs, enzymes, and heriditary material and secondly to be studied as physico-chemical models for biomembranes.

For carrier studies one needs large concentrations of large vesicles. Small vesicles have relatively lower trapping efficiency and some studies indicate that they are also less stable then the large vesicles (23). Stability is another definite requirement for carriers. Ethanolic solution injection method is not suitable for preparing concentrated solutions of vesicles of a definite size and some of the drug to be encapsulated may be removed during dialysis. Thus the

methods using French press cell or polycarbonate membranes may indeed be the methods of choice for preparation of drug carrier vesicles.

For physico-chemical studies, one needs a method that should be able to make vesicles of different sizes of chemically well defined composition. Dialysis of a lipid - detergent mixture is the only other method besides the present method that can accomplish this. However, unless one has dialysed for a very long time, the vesicles will have some detergent or cholesterol dissolved in the membrane phase. Prolonged dialysis may lead to degradation of phospholipid. The vesicles prepared by ethanol injection also have some ethanol trapped in it. However, ethanol being a small molecule is more permeable through the membrane and may be removed more easily and rapidly by gel filtration besides one can vary size maintaining a constant lipid-alcohol ratio. Thus this method is more appropriate for physico-chemical studies. The low concentration of vesicle solution is not a handicap in light scattering studies. If one requires more concentrated solutions, one can concentrate by ultrafiltration. For PMR experiments injection into deutrated solvent followed by concentration will be necessary.

References

1. Finer, E.C., Flook, A.G. and Hauser, H. (1972) Biochim. Biophys. Acta, 260, 49-58.

2. Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, D. and Papahadjopolous, D. (1980), Biochim. Biophys. Acta , 601, 559-571.

3. Barenholtz, Y., Amselem, S. and Lichtenberg, D. (1979), FEBS Letts., 99, 210-214.

4. Hamilton, R.L., Goerke, J., Guo, L.S.S., Williams, M.C. and Havel., R.J. (1980), J. Lip. Res., 21, 981-999.

5. Brunner, J., Skarbal, P. and Hauser, H. (1976), Biochim Biophys. Acta, 455, 322-331.

6. Rhoden, V. and Goldin, S.M. (1979) Biochemistry, 18, 4173-4176.

7. Milsman, M.H.W., Schwenderer, R.A., and Weder, H.G.(1978) Biochim. Biophys. Acta, 512, 147-155.

8. Zumbuehl, O. and Weder, H.G., (1981) Biochim. Biophys. Acta, 640, 252-262.

9. Bazri, S. and Korn, E.D. (1973) Biochim. Biophys. Acta, 298, 1015-1018.

10. Kremer, J.M.H., Esker, M.W.J.v.d., Pathamanoharan, C. and Wesserman, P.H. (1977) Biochemistry, 16, 3932-3935.

11. Deamer, D.W., (1978) Ann. New York, Acad. Sci. 308, 250-253.

12. Szoka, F. and Papahedjopolous, D. (1978) Proc. Natl. Aced. Sci., USA, 75, 4194-4198.

13. Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil. Chem. Soc. 42, 53-56.

14. Cooney, D.O. (1980) Anal. Chem., 52, 1068-1070.

15. Fry, D.W., White, J.C. and Goldman, J.D. (1978) Anal. Biochem. 90, 809-815.

16. Kerker, M. (1969) The scattering of light and other electromagnetic radiations, Academic Press 432-433.

17. Seufert, W.D. (1970) Biophysik. 7, 60-64.

18. Peterson, N.O. and Chan, S.I. (1978) Biochim. Biophys. Acta, 509, 111-128.

19. Martin, F.O. and McDonald, R.C. (1976) Biochemistry 15, 321-327.

20. Aaarts, P.A.M., Gizeman, O.B.J., Kremer, J.M.H., Wasserman, P.H. (1977) Chem. Phys. Lipids 19, 267-274.

21. Tausk, R.J.M., Oudshoorn, C. Overbeek, J. Th.G.(1974) Biophys. Chem. 2, 53-56.

22. Elworthy, P.H. and McIntosh, D.S. (1961) J. Pharm. Pharmacolog. 13, 633.

23. Larabee, A.L. (1979) Biochemistry, 18, 3321-3326.

Éxodos

Chapter V

Addition of Ca^{2+} to a cardiolipin vesicles solution leads to their aggregation, fusion and ultimate precipitation of the Ca^{2+} cardiolipin complex in the hexagonal (H_{11}) phase (1). The kinetics of this process can be followed by turbidity measurements. With higher lipid concentrations stopped flow instrumentation (2) is necessary because turbidity changes may be very rapid. However, with more dilute solutions the kinetics may be followed even with a recording single beam spectrophotometer. An additional advantage of turbidity measurements with dilute solutions is that the data is more interpretable in terms of light scattering theories. I, therefore, decided to follow the kinetics of Ca^{2+} induced turbidity changes in cardiolipin vesicle solutions as a function of added Ca^{2+} concentration to see if one can deduce some information about the phase transition from these measurements, and also the effect of vesicle size on the observed behavior which may be related to the effect of radius of curvature of bilayers on the phase transition characteristics deducible by this method. I also thought it worthwhile to study this phenomenon at different lipid concentrations and temperatures to see if these observations are interpretable in terms of Ca^{2+} binding to cardiolipin alone, or thermotropic behavior also interferes.

Equipped with simple and rapid methods for the isolation and purification of phospholipids, their concentration determination and vesicle preparations I entered the last phase of this work. I thought on the basis of my preliminary experiments that this should be over in no time without any difficulty, but this was not to be so.

To begin with, as observed in the Chapter IV also, the dissymmetry of the cardiolipin vesicle solutions, is greater than that for phosphatidylcholine vesicle solutions prepared under identical conditions. Also when one filters the cardiolipin vesicle solution through membrane filters for dissymmetry measurements, the loss of lipid is more. This indicates that cardiolipin forms larger vesicles. Smaller vesicles can be prepared by decreasing the lipid concentration in the ethanolic solution injected, but this creates sensitivity problems in turbidity and concentration measurements. I initially thought to solve these problems by concentrating the small vesicle solution by ultrafiltration. During some of these preparations I observed that many air bubbles are formed during the course of injection. My earlier experience with the injection method was that the formation of bubbles causes formation of larger vesicles. So in order to avoid it, I degassed the buffer by shaking under vacuo for some time. The

be induced on addition of Ca^{2+} to an aqueous dispersion. A diluter solution was however intact. I tried the degassing experiments again. They showed no change in turbity change characteristics when oxygen is displaced by nitrogen, However, degassing did decrease the turbidity increase rate. For further experiments I isolated fresh lipid, but this time this effect was not observable. I abandoned this project because of pressure of time. I hope to pursue it later.

In order to find out the stability of cardiolipin vesicles vis-a-vis the proposed experiments I observed the turbidity curves of vesicle solutions as a function of time. For large vesicle suspensions (dissymetry greater than 3) the turbidity change in a fixed time interval increases for some time, remains constant for 3-4 hours and then starts decreasing. Also if one tries to determine the phospholipid concentration during this time by the method described in Chapter II, using benzene for extraction, the absorbance of the organic layer at 700 nm decreases monotonically and one can observe an accumulation of the blue complex at the interface (Fig. 1). This effect is independent of the buffer used or the lipid concentration, but is slightly slower at lower temperatures. I then prepared cardiolipin vesicles solutions of much lower dissymetry by a very rapid injection of the ethanolic solution into the buffer.

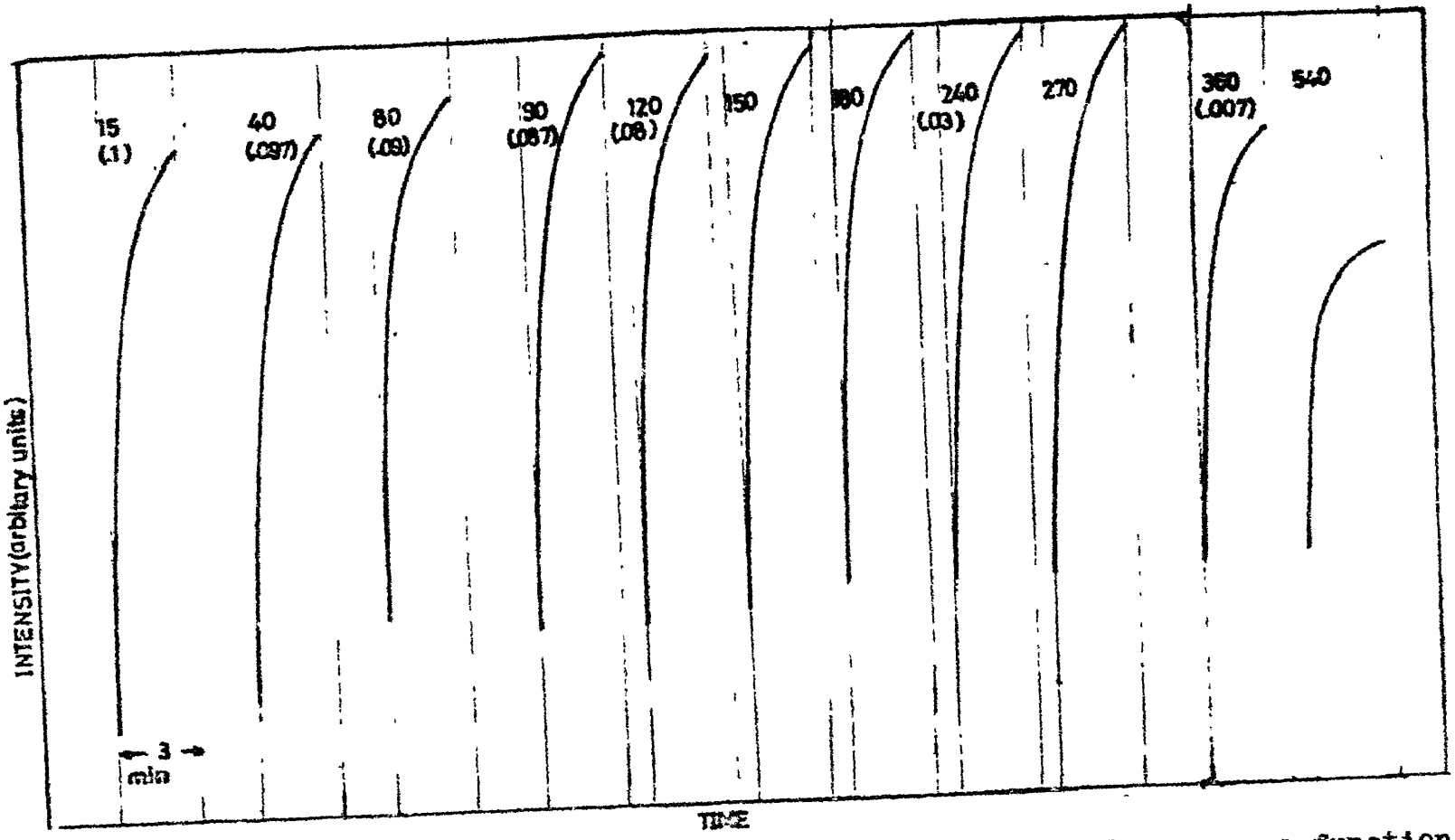


Fig. 1 Rate of change in intensity of transmitted light for 3 min. as a function of incubation time for cardiolipin vesicle solutions on addition of 250 µl. of .4 M $CaCl_2$ to 2.5 ml of lipid dispersion. Conc. = .12 µmole/ml, Dissymmetry = 3.5, temp = $30 \pm 1^{o}C$.
(Figures in bracket indicate the absorbance in the benzene layer of extracted molybdenum complex)

With this solution the above effect is much slower. These conclusions have been confirmed by varying the vesicle size by different changes in the method of preparation. I have not investigated any further the cause of these observations, but I have taken care in my later experiments that a set of observation with a particular vesicle solution is completed before significant degradation may be expected to occur.

These experiments were carried out on a Cary 17D spectrophotometer fitted with a thermostated cell compartment. The lipid used gave a single spot on silicagel TLC with 65:25:4 chloroform-methanol-water (R_f = 0.91). The lipid concentrations were determined using the molybdenum complex method with an extinction coefficient = 0.92 at 30°C using dicholromethane-methanol (8:2) mixture for extraction. All measurements were carried out at 400 nm. Vesicle preparations were made by injecting an ethanolic (or ethanol-water) solution of phospholipid into the aqueous medium (10 mM TES, .05 M NaCl, 2mM Hisidine, pH 7.5). 2.5 ml of the vesicle solution was routinely pipeted into the cuvette and its absorbance checked against a blank of buffer solution before every run. 250μl of $CaCl_2$ solution in the same buffer was added with a microliter pipet and the absorbance changes were observed after 15 seconds of addition of Ca^{2+}. This was achieved with the help of a colleague monitoring time with a stop watch.

The results of these experiments may be summarized as follows :

i) The absorbance increases as function of time in a logarithmic fashion. For low concentrations of Ca^{2+} (less than 2 mM) there is very little turbidity change: However, above a certain threshold concentration of Ca^{2+} ion the rate of turbidity change increases rapidly and beyond a certain concentration of Ca^{2+} (ca 20 mM) the curves obtained by increasing Ca^{2+} concentration further are virtually superimposable within experimental error. Thus the rate reaches a plateau (Fig. 2).

ii) The plot of turbidity change in a fixed time interval vs. the Ca^{2+} ion concentration is largely sigmoidal in shape. There are some deviations from a simple sigmoidal shape in the case of solutions containing multilamellar liposomes: The Ca^{2+}/lipid ratio at the point of maximum slope is about 100 and it does not change significantly with the lipid concentration in the range 10-30 μM, (Fig. 3).

iii) For vesicles of very different sizes (i.e. 1:10) at the same lipid concentration although the turbidities at the plateau region are identical, the absolute change in turbidity are significantly different. Thus, Δ A is always lower for larger vesicles (Fig. 4).

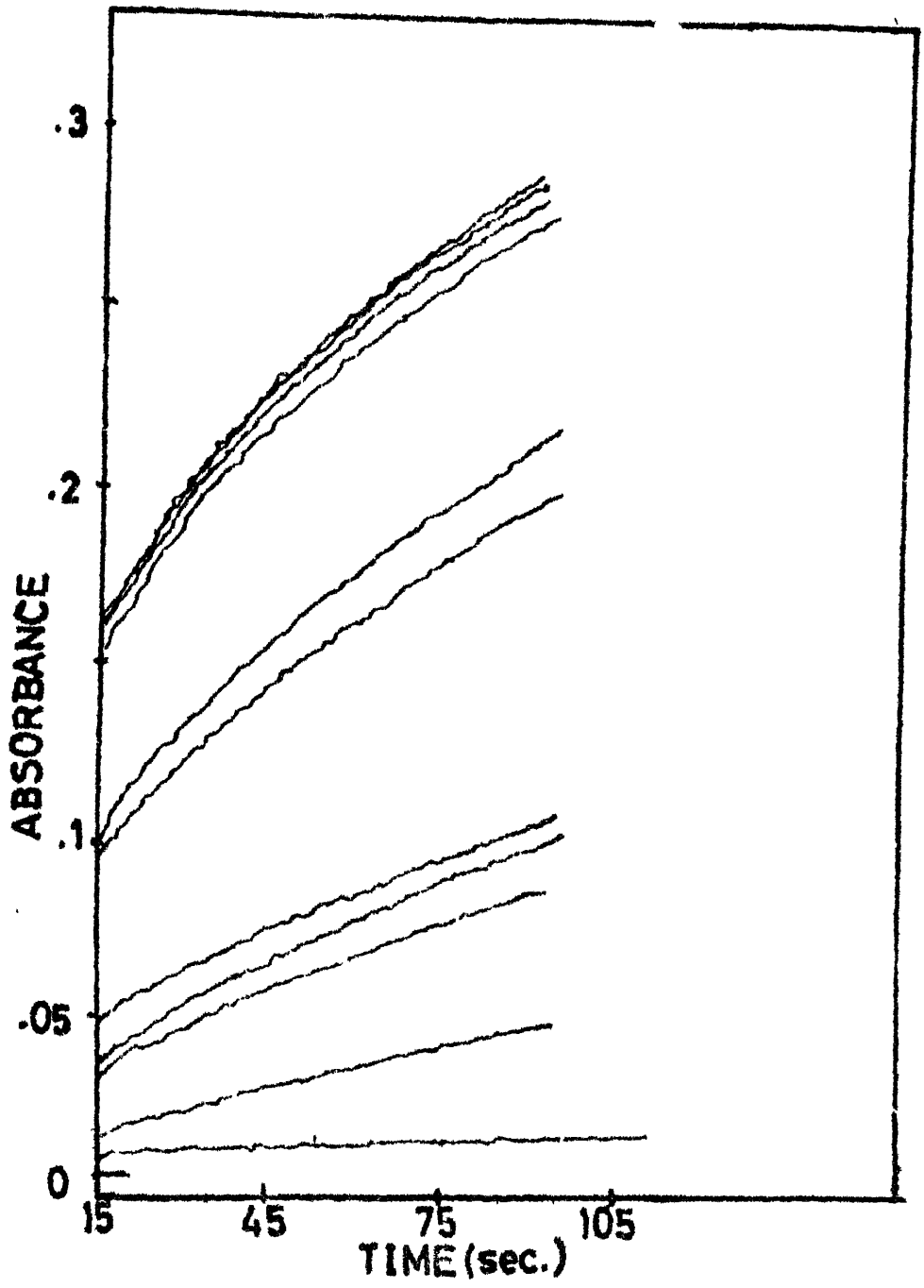


Fig. 2 A typical set of turbidity change curves for cardiolipin vesicles, conc. = .08 μmole/ml, Dissymmetry = 1.6

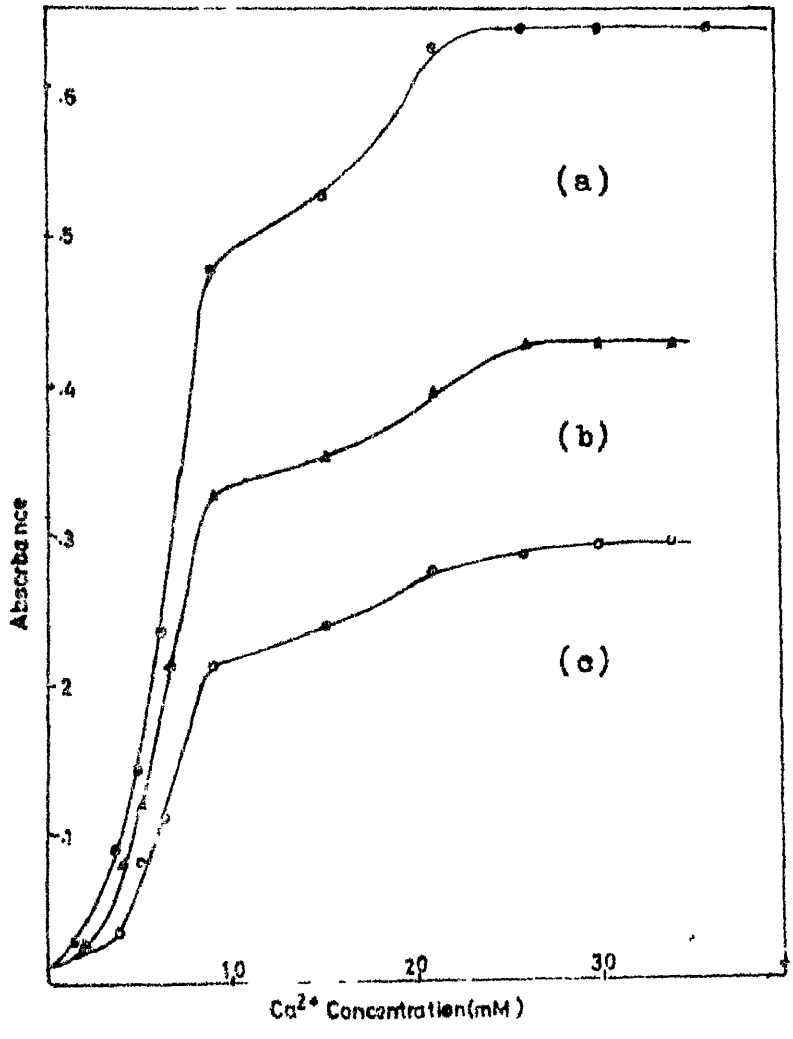


Fig. 3 Absorbance change in 75 sec. as a function of Ca^{2+} i concentration for cardiolipin vesicles dispersions of dissymmetry = 1.6 and temperature = 30 ± 1°C, lipid conc. (a) .0507, (b) .0383, (c) .0286 μmole/ml.

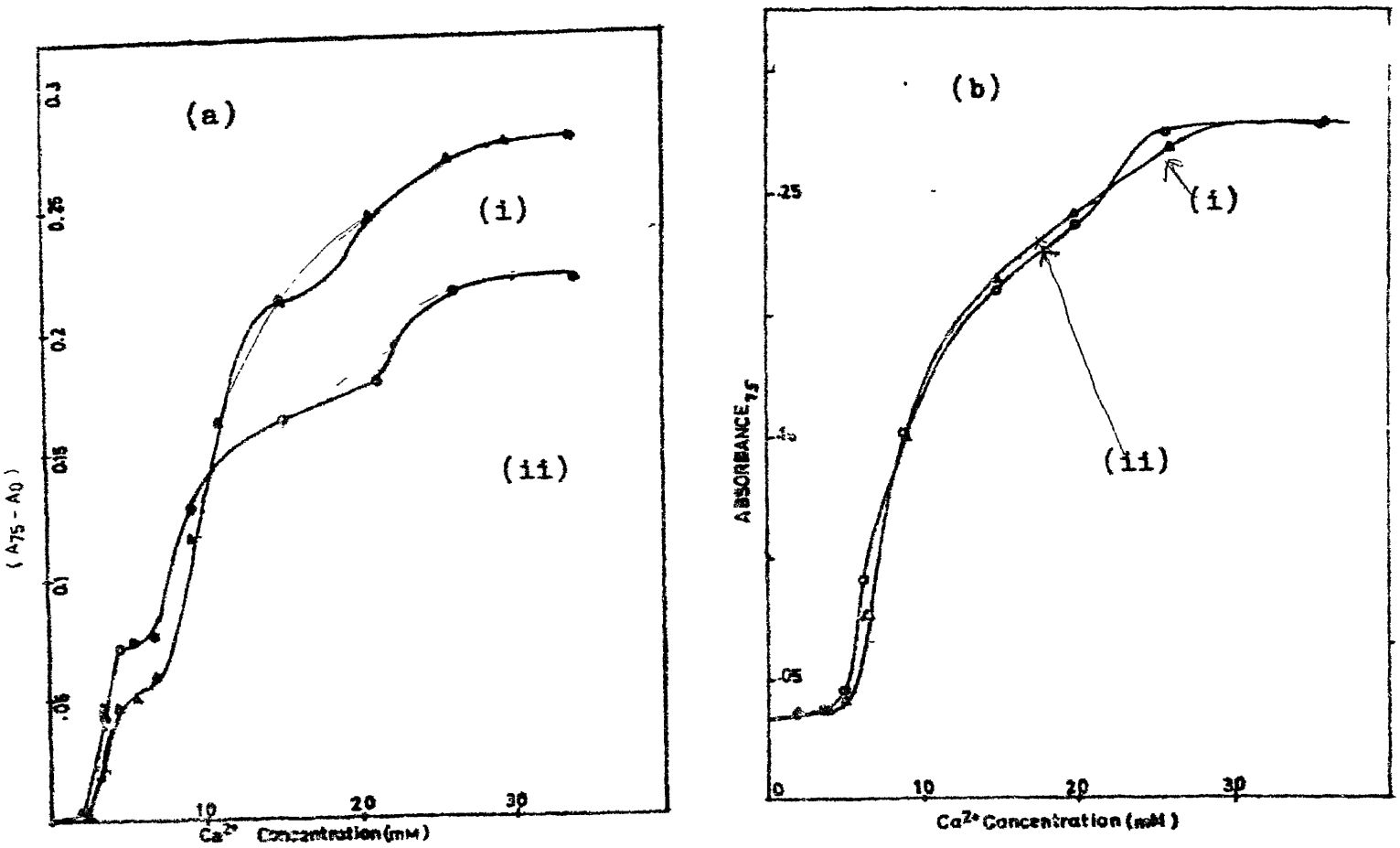


Fig. 4 Absorbance change in 75 sec. as a function of Ca^{2+} concentration for cardiolipin vesicles of (a) conc = .037 μmole/ml, temp. = 30 ± 1°C, for two unfiltered preparations, (i) prepared by rapid injection of ethanolics solution, (ii) prepared by injection of 10:2 ethanol-water solution of lipid in the buffer (b) conc. = .030 μmole/ml, temperature = 30 ± 1°C, for two filtered vesicles solutions. Dissymmetry (i) = 1.2, (ii) 2.1.

iv) With vesicle solutions of almost equal vesicle concentration of different sizes and therefore different lipid concentration, the A vs Ca^{2+} curve for larger vesicles is above that for smaller vesicles. (Fig. 5).

v) There is no appreciable difference in the shape of the sigmoidal curve, when the experiment is carried out with a vesicle solution at 30°C and 20°C. However, I observed that any turbidity change curve shifts upwards with larger slope as a function of the incubation time for about 90 minutes at 15°C (Fig. 6).

These results may be interpreted as follows :

For a non absorbing system which scatters light, the turbidity is defined as

$$\tau = -\ln\left(\frac{I}{I_o}\right) = 2.303\ A \qquad (1)$$

where I_o is the intensity of the incident light. I is the intensity of the transmitted light, and A is the absorbance measured. The reciprocal of the turbidity is the length of the turbid medium required to reduce the intensity of a light beam to e^{-1} of its initial intensity. It is a measure of light scattered at all angles. The turbidity can be related to the molecular weight of the scattering particles by the following relation

$$\tau = -\ln\left(1 - \frac{16\,\pi\,kcMQ}{3}\right) \qquad (2)$$

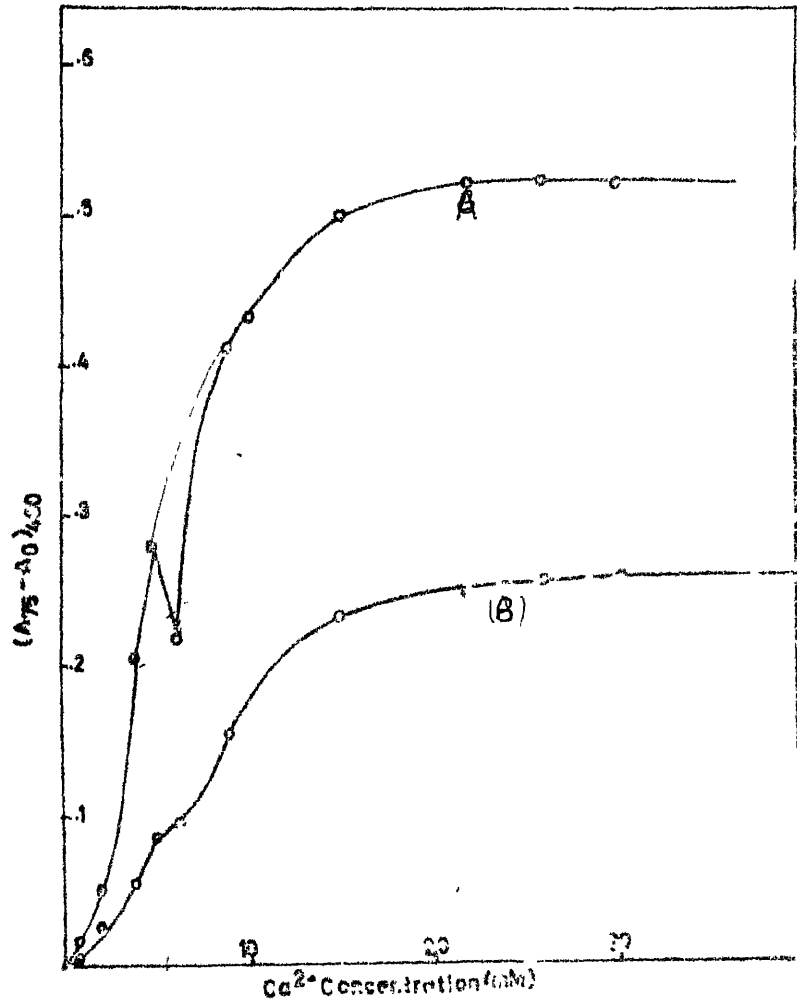


Fig. 5 Absorbance change in 75 sec. as a function of Ca^{2+} for cardiolipin vesicles solutions at 20°C, for equal vesicle conc. calculated from concentration and dissymmetry $R_A/R_B = 1.4$, i.e., $C_A/C_B = (R_A/R_B)^2 = 2$

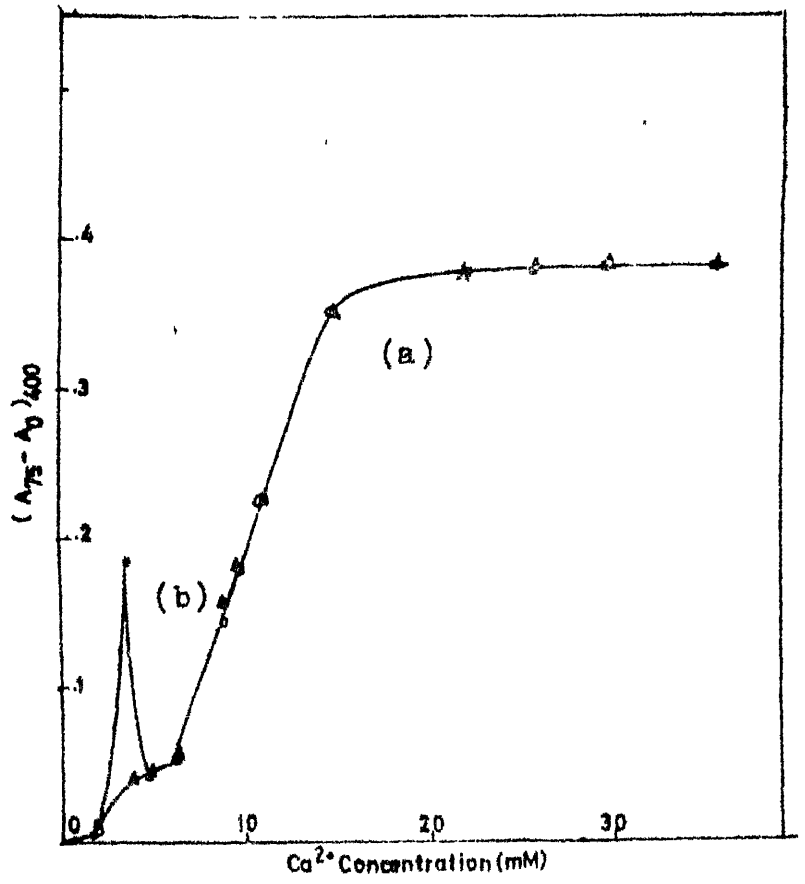


Fig. 6 Absorbance change in 75 sec. as a function of Ca^{2+} for cardiolipin vesicles solutions at 30°C and 20°C

For small values of turbidity, this may be approximated by

$$\tau = \frac{16\pi KcMQ}{3}$$

K is a constant given by $K = \frac{2\pi^2 n_o^2 \varphi^2}{N_o X^4}$ (3)

where n_o is the refractive index of the solvent, φ is the rate of change of refractive index, with concentration. N_o is the Avagadro's number, X is the wave length of the incident light, M is the molecular weight of the scattering particle and Q is the particle dissipation factor which takes into account intra-particles scattering. The approximation is valid only if the absorbance is lower than 0.1 cm^{-1}.(3).

Let us first of all consider the A vs. Ca^{2+} curves with a radius ratio of 1:1.4. (fig.5) Here the lipid concentrations are different and since the absorbance values are above .1 cm^{-1} one has to use relation (2) to calculate the product KMQ for a given concentration. In Table I we present the relative values of KMQ for the large and the small vesicles.

If the small and the large vesicles were equally aggregration and fusion susceptible, the molecular weights of the fused larger vesicles would be twice the smaller. If we leave out the complication due to particle scattering factor Q for the time being and assume that any differences in the values of K at different concentrations are minimal at the low concentration used in these experiments, then our data indicates

that the smaller vesicles give rise to larger molecular weight particles under similar conditions. This would imply that the smaller vesicles are more fusible since larger vesicles other wise have a larger cross sectional area besides higher molecular weight.

But there is a word of caution here. Taking into account the complication due to particle scattering factor can complicate and upset this conclusion. Particle scattering factor decreases with size and changes with structure. Thus turbidity does not always increase with molecular weight (4). But the conclusion, that is inescapeable, is that there is difference in fusion rate between smaller and larger vesicles.

The complication due to Q can even upset the interpretation of the A vs. Ca^{2+} curve. If turbidity changes monotonically with M the simple interpretation of the curve is that the (aggregration) fusion rate increases with the Ca^{2+} concentration above a threshold sharply and then levels off. This conclusion has been reached by Lansman & Haynes (2) also. From their stopped flow turbidity measurements they have concluded that this kind of behavior may be explained in terms of a dimerization mechanism. Perhaps their turbidity changes are much smaller and hence the interpretation is not complicated by Q factor. We observe a similar behavior and thus in the line of their

interpretation we conclude pending further investigations, that at identical total Ca^{2+} concentration small vesicles fuse more efficiently than larger vesicles.

Now let us consider observation (ii). If the abrupt change in turbidity rate was either due to precipitation or aggregation, one would expect a correlation between Ca^{2+} and lipid concentrations. However, this is not observed. In fact, the Ca^{2+}/lipid conc. ratio required for these abrupt changes is about 100 times than that observed by other workers with cardiolipin and other acidic phospholipids (5). Also the concentration of Ca^{2+} required for such changes at very different lipid concentrations fall in a very narrow range.

In Table II we present the KMQ values at different Ca^{2+} concentrations. However, we are unable to draw any meaningful conclusion from them.

It is known, in the studies on phase transitions (6), that when one phase is changed into another, the new phase, although thermodynamically stable, appears in the form of nuclei only when considrable supersaturation or super-cooling has been achieved. Surface phenomenon play an important role in phase changes. The usual thermodynamic

Table 1

Relative KMQ values after 75 seconds of addition of Ca^{2+} to cardiolipin vesicle solutions of different size distribution but similar vesicle concentration, calculated from data in Fig. 5. Initial dissymmetry for B = 1.6 and A = 2.0

Ca^{2+} conc.	Relative KMQ	
	B	A
2	0.0068	0.0068
4	0.028	0.038
6	0.042	0.039
9	0.064	0.053
15	0.082	0.056
22	0.086	0.057

Table 2

Relative KMQ values after 75 seconds of addition of Ca^{2+} to cardiolipin vesicle solutions of different lipid concentration, but some size distribution, calculated from data in Fig. 3

Ca^{2+} concentration mM	Relative KMQ		
	a	b	c
4	0.022	0.03	0.0176
6	0.0516	0.072	0.0548
9	0.0832	0.0978	0.0952
15	0.089	0.107	0.105
21	0.094	0.087	0.148
26	0.0912	0.117	0.126

theory of phase transitions deals not with the course of these transitions i.e. the velocity with which they progress under given conditions but with the conditions under which the velocity is zero. The growth of a new phase B, after it has made its appearance can proceed at the cost of the initial phase A only if they are not in equilibrium with each other. This deviation from equilibrium can be very minute when the new phase is sufficiently developed. Much larger deviations are however necessary, at the initial stage of development of the new phase, in the form of embryonous 'nuclei', i.e. a very small element of volume that has suffered the phase transition.

The nucleation phenomenon has been studied widely for various phase changes (7). One of the earliest theoretical formulations of this process is due to Volmer (8), which has been further developed by Becker and Döring (9). In the kinetics of various phase transitions, behavior very similar to our observation is observed. Thus we can try to interpret our results in terms of the nucleation theory. Ca^{2+} is known to form bridges between vesicles of acidic phospholipids and cause an instability of the bilayer (10). These bridges can only be formed in a binary collision of two vesicles with a Ca^{2+} in the line of collision. Thus on addition of Ca^{2+} aggregation i.e. formation of clusters ensues. The phase transition may then be expected to occur

with a critical cluster size.

The turbidity changes in our experiments are perhaps due to the growth of small domains of the hexagonal phase from the critical clusters. Thus the rate of the turbidity change will depend on the concentration of the critical clusters. In dilute vesicle solutions, since the collision frequency will be lower and the concentration of the critical clusters will also be lower at a given Ca^{2+} concentration than in a more concentrated solution. The fusion probability also may be expected to depend on the Ca^{2+} loading of the vesicles. The binding constants of Ca^{2+} to acidic phospholipids are known to decrease with increasing Ca^{2+} concentration (11). Thus, for the formation of a certain concentration of critical clusters, in a certain time, one will require a much higher Ca^{2+}/lipid ratio for more dilute vesicle solution. This explains qualitatively observation (ii).

The observation that regardless of size, the turbidity change rates above a threshold concentration of Ca^{2+} are equal suggests that the turbidity observed is due to very similar particles, e.g. crystallites only.

From a structural viewpoint, the effect of vesicle size can be related to the packing differences discussed in Chapter I. The packing influences the surface tension, hence the critical size of clusters required for different vesicle sizes is also expected to be different. If our earlier

conclusions are correct then for smaller vesicles, the critical cluster size must be larger.

The ΔA vs. Ca^{2+} curve for unfiltered vesicle solutions (Fig. 4a) which is expected to contain some multilameller vesicles shows some deviations from the sigmoidal curve. This can be due to some more complex phenomenon in the nucleation process of these solutions. The discontinuities observed in some other curves are also not easily explainable.

The peculiar behavior at 15°C may be because of some time dependent thermotropic transformations in the vesicle solutions. However, since we do not have adequate data on the thermotropic phase behavior of the lipid used no definite conclusions can be drawn.

An interesting offshoot of our work is that one can also explain the recent observations of an increase in the fusion rate of phosphatidylserine vesicles by Ca^{2+} in the presence of calcium phosphate (12) in terms of hetrogeneous nucleation.

References

1. Vail, W.J., and Stollery, J.G. (1980), Biochim. Biophys. Acta, 551, 74.

2. Lansman, J. and Haynes, D.A. (1975) Biochim. Biophys. Acta, 394, 335.

3. Peterson, N.O., and Chan, S.I. (1978), Biochim. Biophys. Acta, 509, 111.

4. Chong, C.S. and Colbow, K. (1976), Biochim. Biophys. Acta, 436, 260.

5. Cullis, P.R. and Kruijff, B. (1979), Biochim. Biophys. Acta, 559, 399.

6. Frenkel, J. (1946), Kinetic Theory of Liquids, Oxford University Press, N.Y. Chapter VII.

7. Bradley, R.S. (1951) Quart. Revs. Lond. 5, 315.

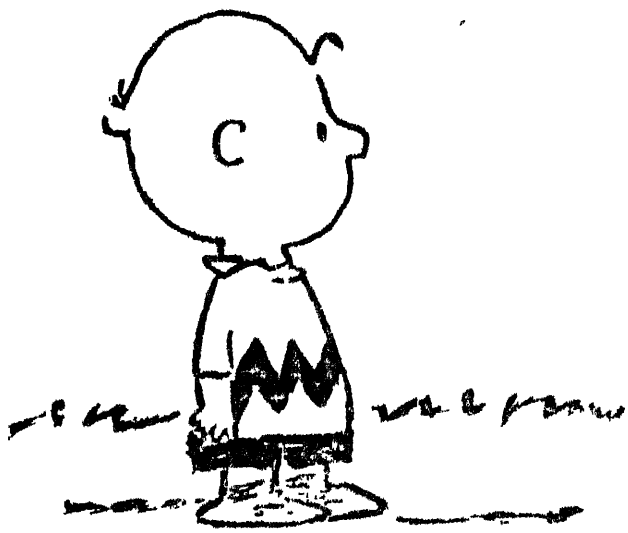
8. Volmer, M. (1939), Kinetic der Phasenbildung, Steinkopf.

9. Becker, R. and Döring, W. (1935), Ann. Physik, 24, 719.

10. Portis, A., Newton, C., Pangborn, W. and Papahadjopolous,D. (1979), Biochemistry.

11. Papahadjopolous, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R., (1977), Biochim. Biophys. Acta, 465, 579.

12. Fraley, R., Wilschut, J., Duzgunes, N., Smith, S., and Papahadjopolous, D., (1980), Biochemistry, 19, 6021.



Next to nothing for weight,
And since they grew duller
From contact with earth
Next to nothing for color,
Next to nothing for use,
But a crop is a crop
And who's to say where
The harvest shall stop.

Robert Frost (1923)

Epilogue

I feel a bit uneasy. This is not all that I had expected in my thesis. I wanted to study the binding of Ca^{2+} to cardiolipin vesicles using metallochromic indicators and study some mixed phospholipid systems also. In fact, I have ideas for an elegant study of the miscibility of different phospholipid with each other in the vesicles, from which one can decide whether when one makes mixed PC + CL vesicles, for example, all the vesicles will contain these lipids in the same proportion, or is there a predisposition for the formation of vesicles of different compositions. But then there were factors beyond my control. One had to sit almost idle for months together, waiting that the power supply will be predictable. The Toshniwal spectrophotometer, I planned to use, started giving all kinds of problems, only when I started my work, and then there were those unpredictable observations. Perhaps if things were different I could have done more and better.

I have a hypothesis which can explain some of the earlier unreproduced observations in Chapter V. The observation that the molybdenum complex was not extractable into the benzene layer, can only be explained by it being more polar, since it can be extracted with slightly more polar solvents. This is possible in the present system if the lipid undergoes

either hydrolysis, as was the case on the storage, or if it undergoes oxidation. Hydrolysed lipid as observed with the degraded stored lipid, would not show any appreciable turbidity change, on addition of Ca^{2+}. The turbidity change decreases after longer standing also, which can be indicative of hydrolysis. But the initial rise in turbidity change and its constancy could very well be due to an oxidation of the cardiolipin in bilayer vesicles by the oxygen dissolved in the bilayers. Some studies on solubility of various gases in the lipid bilayer have been reported in the last few years. The reason we could not reproduce our earlier results can be simply due to greater degree of autooxidation of the second sample which is not detectable on TLC. If this is plausible then one needs a very careful experimentation with cardiolipin vesicle using oxidation of the side chain as a parameter. The results of such a study would be far reaching in our understanding of respiratory processes of mitochondria.

I hope I will be able to do these studies some day, and if my speculation, then comes out to be true, I may say I had done something in this thesis.

Vitae

I am 28 years old now. I did B.Sc. (H) Chem. and M.Sc. (Physical Chem.) from University of Delhi in 1974 after which I joined the graduate program at IIT Kanpur. I am still unmarried, waiting for someone to fall in love with me. I want to win the Nobel Prize and write a Bestseller.